SELENIUM SUPPLEMENTATION IN CATTLE:

TRANSFER TO MILK AND EFFECT ON UDDER HEALTH

BY

ALEJANDRO CEBALLOS-MARQUEZ

A Thesis

Submitted to the Graduate Faculty.

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Health Management

Faculty of Veterinary Medicine

University of Prince Edward Island

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Abstract

Soils in many regions of the world have low selenium (Se) content due to an uneven distribution of Se in the earth's crust. Selenium deficiency has been associated with a wide range of costly disorders in cattle, such as retained placenta, lowered milk production, reduced growth rate, altered immune responses and reduced disease resistance. Mastitis is likely to provide the most convincing evidence for the influence of Se on disease resistance. The aim of this thesis was to evaluate the effect of Se status and Se supplementation on milk Se concentration, and on indicators of udder health. A systematic review and meta-analysis of the effect of oral Se supplementation on milk Se concentration in cattle indicated that milk Se concentration increased, on average, 0.16 μmol/L, with a significant heterogeneity among studies. North American cows supplemented with Se yeast had higher milk Se concentration when measured approximately 75 d after the beginning of supplementation. This information provided the basis for tailoring daily animal requirements in order to enhance Se intake of consumers of dairy products. Mean cow-level milk Se concentration was considered marginal in 14% of dairy cows from Atlantic Canadian dairy herds. The risk of having a marginal Se status was highest when cows were pastured during the grazing season. Selenium status was not associated with the overall odds of new intramammary infections (IMI) in the dry period. However, new IMI caused by *Streptococcus* spp. and by other Gram-positive pathogens increased with higher milk Se concentration. Bulk tank milk Se from Canadian herds was not associated with milk production parameters or bulk tank somatic cell count (BTSCC). However, higher values of BTSe were associated with lower risk of being a *Staph. aureus*-positive herd. In pastured herds in Chile, whole blood activity of glutathione peroxidase (GPx) increased after Se supplementation beginning 45 days before calving, no matter what source of Se was used. Supplementation did not affect udder health in the subsequent lactation in multiparous cows, indicating that dietary basal Se intake (approximately 20% of the current recommendation) was adequate for preventing subclinical mastitis in pasture-based cows in Southern Chile. The effect of prepartum supplementation with a single injection of barium selenate or organic Se on the risk of IMI and SCC around calving in pastured first-lactation dairy heifers was studied in Chilean dairy herds. Supplementation was accompanied by a reduction in the prevalence of IMI and SCC at calving, but did not affect the risk of new IMI and SCC in milk during the balance of the first month of lactation. In conclusion, milk Se concentration does not appear to be a principal determinant of udder health in Atlantic Canadian dairy herds, or milk production parameters in Canadian dairy herds. However, higher values of BTSe may be associated with lower risk of being a *Staph. aureus*-positive herd. In cows raised in pastoral systems in Southern Chile, an intake of approximately 1 mg Se/cow/d was adequate for preventing subclinical mastitis during lactation. However, pre-calving Se supplementation appears beneficial for preventing subclinical mastitis shortly after calving in heifers grazing low Se pastures.

Acknowledgements

Selene and Endymion (Nicolas Poussin, c. 1630)

In loving memory of my parents

Monica and Federico, now it is your turn!

Yo no estudio para escribir, ni menos para enseñar (que fuera en mí desmedida soberbia),

sino sólo por ver si con estudiar ignoro menos. Así lo respondo y así lo siento.

Sor Juana Inés de la Cruz (Respuesta de la poetisa a la muy Ilustre Sor Filotea de la Cruz, 1691)

[I do not study in order to write, nor far less in order to teach (which would be boundless

arrogance in me),

but simply to see whether by studying I may become less ignorant. This is my answer, and these

are my feelings.]

Sor Juana Inés de la Cruz (Poet's reply to the most Illustrious Sister Filotea de la Cruz, 1691)

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LIST OF ABBREVIATIONS

AA	Apparent absorption
ARC	Agricultural Research Council
cfu	Colony forming unit
BTSe	Bulk tank milk selenium concentration
BMSCC	Bulk tank milk somatic cell count
CBMRN	Canadian Bovine Mastitis Research Network
CI	Confidence interval
CM	Clinical mastitis
CNS	Coagulase-negative staphylococci
d	Day(s)
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
GEE	Generalized estimating equations
GPx	Glutathione peroxidase
IMI	Intramammary infection
IRR	Incidence rate ratio
LnSCC	Natural log of somatic cell count
LW	Live weight
mo	Month(s)
NB	New Brunswick
NMC	National Mastitis Council
NRC	National Research Council
NS	Nova Scotia
OMI	Organic matter intake
OR	Odds ratio
PMN	Polymorphonuclear cells
ppm	Parts per million
PEI	Prince Edward Island
PMN	Polymorphonuclear cells
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SCC	Somatic cell count
SE	Standard error
Se-cys	Selenocysteine
Se-met	Selenomethionine
TA	True or net absorption
TrxR	Thioredoxin reductase
wk	Week(s)

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1 GENERAL INTRODUCTION

Selenium (Se, named after Σελη 'νη, the Greek goddess of moon) is a naturally occurring solid substance, which can be found in several forms ranging from a red powder to gray black crystals, with atomic number 34 and an atomic mass of 78.96. Selenium belongs to the group VIA of the periodic table of the elements, is located between sulfur and tellurium, and resembles sulfur both in its various forms and in its compounds. Selenium is typically defined as nonmetallic, and is widely but unevenly distributed in the crust of the earth (EMEA, 1999). The toxicity of Se has been described since the 1930s; however, it was described to be an essential nutrient for mammals almost 50 years ago (Schwarz et al., 1957; Schwarz and Foltz, 1957; Schwarz and Foltz, 1958). As an essential trace element, Se serves several purposes. It is an integral component of a variety of enzymes, such as glutathione peroxidase (GPx) (Flohe et al., 1973; Rotruck et al., 1973), and has an important role in immunological and physiological aspects (Boyne and Arthur, 1981; Arthur et al., 2003; Bianco and Larsen, 2006).

Soils in many regions of the world have low Se content, typically found at concentrations between 0.1 and 2 ppm, with only a small portion available to the plants. As a consequence, forages and crops grown on these soils may provide inadequate dietary Se for grazing animals and humans. Although Se is not an essential nutrient for plant growth, some agricultural practices (i.e. fertilization) may reduce the Se content in soils and plants (Hartikainen, 2005). The consumption of animal products (e.g. milk) derived from animals grazing low-Se areas, can influence the Se status of entire human communities, putting humans at risk of overt deficiency (Combs, 2001). Several countries have recognized this risk, such as Finland, where Se application to grain crops grown for human consumption is required by law and has resulted in a significant improvement in Se status of the population (Hartikainen and Ekholm, 2001).

Selenium deficiency in dairy cattle has been related to a wide range of disorders reviewed by Wichtel (1998a). Retained placenta, infertility, lowered milk production, reduced

growth rate, altered immune responses and reduced disease resistance, have been associated with deficiency. When considering antioxidant-responsive disease in cattle, vitamin E and Se are often considered together. This is because vitamin E and Se appear to work in concert as cellular antioxidants, can have a sparing effect on each other with respect to their protective effects in some diseases (e.g. retained placenta and mastitis), and are often administered together in the same formulation (Wichtel, 1998a).

Over time, the beneficial effects of Se supplementation have been identified in several investigations, and supplementation is needed in those regions where Se in soils, forages and crops is low. Feeding systems and parenteral delivery methods to increase Se status in cattle have been developed. In fact, many experiments have shown that Se supplementation results in an increase of Se content in body tissues and milk; however, that Se content does not appear to increase linearly as Se intake increases (Conrad and Moxon, 1979; Maus et al., 1980; Aspila, 1991). Further, the type of Se supplement (i.e. inorganic or organic) appears to influence the degree of accumulation of Se in blood, tissues and milk (Knowles et al., 1999; Weiss, 2005). Additionally, results of trials conducted in New Zealand did not support the contention that Se supplementation results in an improvement of the udder immune response (Whelan et al., 1992; Wichtel et al., 1994; Grace et al., 1997).

1.1   Distribution of Selenium

The source of Se in any food system resides in the rocks and soils of the terrestrial environment, in which it is ubiquitous but not evenly distributed (Oldfield, 2002). Concentration in most soils falls between 0.1 and 2 ppm of Se, ranging from practically none up to 100 ppm. Soils containing < 0.6 ppm of Se are considered deficient (Gupta and Gupta, 2000). Some parts of the world (e.g. the Nordic countries, New Zealand, China, and the Pacific and Atlantic coast

of Canada) are notable for having very low amounts of Se in their soils and, therefore, their food systems. In contrast, other areas (e.g. the Great Plains of the United States of America and Central Canada) have a higher content of Se in soils (Combs, 2001). Selenium is removed from soil by plants, and then incorporated into their tissues as selenocompounds (Terry et al., 2000; Whanger, 2002).

The Pacific and Atlantic coasts of Canada are known to contain unusually low concentrations of Se, while Central Canada may be largely adequate (Oldfield, 2002). Early surveys conducted in British Columbia (Fenimore et al., 1983) and Atlantic Canada (Winter and Gupta, 1979) have found that most of forages grown on those regions have a mean Se level < 0.05 ppm. Approximately 90% of forage samples in Atlantic Canada contained < 0.05 ppm of Se (Winter and Gupta, 1979). In Ontario, a lower number of forage samples (~ 50%) had < 0.05 ppm (Young et al., 1977). Studies in British Columbia have found marginal serum Se levels in cattle fed with Se-deficient hay (Fenimore et al., 1983). Data collected from beef farms on PEI showed that local forages provide inadequate Se for cattle (Vokaty, 1991). More recently, a survey on Se status in dairy herds from Prince Edward Island found that many producers were providing insufficient supplementary Se to meet the recommended intake for dairy cattle (Wichtel et al., 2004). Taken together, these results indicate that Se deficiency in livestock is widespread in Canada, particularly in the coastal provinces.

1.2   Metabolism of Selenium in Cattle

1.2.1   Absorption

The available literature on Se metabolism in ruminants is not as ample as seen in non-ruminants, and most of the information has been extrapolated from data obtained from

laboratory animals. However, such extrapolation may be problematic because of extensive

ruminal metabolism of Se that occurs in ruminants (Weiss, 2005).

Sodium selenate or selenite, and organic forms (e.g. selenomethionine) coming from the

basal diet or selenized yeast (Se yeast) are the most commonly used forms of Se

supplementation (Ullrey, 1992); however, they differ in their metabolism in ruminants, and

several factors affect the efficiency of Se absorption including:

    i.   The chemical form of the element;

    ii.   The amount ingested, and the status prior to supplementation;

    iii.   The presence of other dietary factors, such as antagonists (e.g. As, Ca, Co, Fe).

NRC (2001) indicated an apparent digestibility of Se in forages and concentrates ranging

between 30 and 60%; this variation is likely to be related to a great degree to metabolic changes

of Se in the rumen. Most of selenate consumed is reduced to selenite in the rumen, and a portion

leaves the rumen, being absorbed as selenate in the small intestine, probably via an active

transport system (Paulson et al., 1968; Weiss, 2005). Selenite is reduced to low molecular forms,

and some is used to synthesize selenoamino acids, predominantly selenocysteine (Se-cys), which

are incorporated into microbial protein. The remaining selenite escapes the rumen, and is

absorbed in the small intestine, more efficiently in the duodenum than in the jejunum or ileum,

via a passive mechanism. Insoluble Se forms are not utilized by the host, and are excreted via

feces (Weiss, 2005).

The coefficient of true absorption (TA) is the difference between the dietary intake and

fecal excretion after the correction for endogenous fecal losses, while the apparent absorption

(AA) corresponds to the difference between the intake and fecal excretion (Table 1.1). The

efficiency of Se absorption in ruminants is variable and the information is not extensive, but Se

absorption is now accepted to be lower in ruminants than in monogastrics. Therefore, these

discrepancies might be related to a systematic overestimation of Se intake or a systematic underestimation of fecal Se (Krishnamurti et al., 1989).

When organic forms are supplemented (i.e. Se yeast), Se from selenoamino acids appears to undergo fewer alterations in the ruminal environment than selenate or selenite (Weiss, 2005). Selenomethionine (Se-met) is the major Se compound in Se yeast (Połatajko et al., 2004); therefore, Se-met acid is probably absorbed from the intestine ($\sim$ 90%) via the methionine transporter system (Weiss, 2005). Even though data about the digestibility of Se-met are limited and highly variable between studies, experiments carried out with small ruminants have indicated coefficients ranging between 40% (Koenig et al., 1997) and 60% (Aspila, 1991) for the TA of organic Se. Further, Weiss (2005) estimated an apparent digestibility of 66% for organic Se, which is approximately 40% higher than the apparent digestibility of Se from inorganic forms (Weiss, 2005).

1.2.2   Metabolic Transformation and Excretion

The metabolic function of Se has been widely studied, in particular, its incorporation into selenoproteins (e.g. antioxidants). To date, several investigations have strengthened the idea that the essential role of Se in biology is due to its presence in proteins in the form of Se-cys, controversially known as the 21st amino acid (Longtin, 2004; Gladyshev, 2006).

The liver is an essential organ in Se metabolism (Symonds et al., 1981b). Several metabolic reactions occurs in hepatocytes where Se is transformed into compounds which are available for other organs, and through which Se is excreted from the body (Behne and Hofer-Bosse, 1984; Aspila, 1991).

Table 1.1. Coefficient of absorption of inorganic selenium in ruminants according to the physiological status.

Species	Status[1]	Abs.[3]	Range (%)[4]	Reference
Cattle	Dry	AA	17 - 50	Harrison and Conrad (1984a)
	Dry	AA	26 - 40	Harrison and Conrad (1984b)
	Dry	TA	11	Koenig et al. (1991a)
	Dry	TA	10 - 16	Koenig et al. (1991b)
	Lactation	TA	32	Symonds et al. (1981b)
	NS[2]	TA	40	Grace (1992)
	NS	TA	< 40	Ivancic and Weiss (2001)
Sheep	Not pregnant	AA	34 - 55	Krishnamurti et al. (1989)
	Not pregnant	TA	50	Krishnamurti et al. (1989)
	Not pregnant	TA	18 - 53	Krishnamurti et al. (1997)
	NS	AA	29	Wright and Bell (1966)
Goats	Lactation	AA	31 - 61	Aspila (1991)

[1]Physiological status.
[2]Not specified.
[3]Absorption (TA: true absorption. AA: apparent absorption).
[4]Range for the coefficient of absorption.

After absorption, Se is rapidly taken from the plasma by the liver with a subsequent return to plasma in a form whose kinetics is different from dietary Se (Krishnamurti et al., 1989). After absorption, selenite goes into cells and is reduced to hydride selenide, which is later used to synthesize Se-met (Weiss, 2005). When Se comes from organic sources, Se-met enters the general protein metabolic pool as a mimic for methionine, being well retained in tissues (Combs, 2001). In the case of Se-cys, a reduction to hydride selenide is first required, and then used for Se-enzyme synthesis, and less is retained in tissues (Combs, 2001). If Se-cys is absorbed from the diet, it cannot be inserted directly into the active site of the selenoenzymes because it does not have the specific tRNA to determine the position of insertion (Weiss, 2005). The incorporation of Se into specific selenoproteins eases transfer to fetal tissue, allows for increased incorporation into milk, and makes possible a reserve supply as muscle cells are replaced.

The biological functions of Se are thought to be mediated through at least 25 selenoproteins with known functions, where Se-cys is required in redox catalysis, and not all of them act as part of the antioxidant system (Wichtel, 1998a; Gladyshev, 2006). The description of selenoproteins has motivated the publication of several reviews (Behne and Kyriakopoulos, 2001; Hatfield et al., 2006; Lu and Holmgren, 2009).

The regulation and synthesis of selenoproteins is affected by suboptimal Se intake, and each selenoprotein is affected differently (Wichtel, 1998a; Lu and Holmgren, 2009). Moreover, a lack of methionine in the diet also affects the metabolism of selenoamino acids. When the intake of methionine is a limiting factor in the diet, a significant increase in the percentage of Se-met is incorporated into nonspecific body proteins for methionine because the methionine-tRNA cannot distinguish between methionine and Se-met (Whanger, 2002). The biological function of some selenoproteins is summarized in Table 1.2.

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Table 1.2. Biological function of some selenoproteins that occur in vertebrates and their biological function.

Selenoprotein	EC[1]	Function	Reference
GPx[2,*]	1.11.1.9	Antioxidant properties Regulation of inflammatory response	Flohe et al. (1973), Rotruck et al. (1973), Arthur (2000)
DIO[3]	1.97.1.10	Deiodination of thyroxine	Bianco and Larsen (2006)
TrxR[4,*]	1.8.1.9	Reduction of thioredoxin Antioxidant properties	Gladyshev (2006)
SelP[5]		Selenium homeostasis Antioxidant properties	Burk and Hill (2005), Gladyshev (2006)
SelW[6]		Unknown Muscle metabolism (?)	Yeh et al. (1995), Behne and Kyriakopoulos (2001), Gladyshev (2006)

[1]Enzyme Commission Number (International Union of Biochemistry and Molecular Biology).
[2]Glutathione peroxidase.
[3]Thyroid deiodinases.
[4]Thioredoxin reductases.
[5]Selenoprotein P.
[6]Selenoprotein W.
*Expressed also in the bovine mammary gland (Bruzelius et al., 2007; Sordillo et al., 2007).

The response of selenoproteins (e.g. GPx) to Se deprivation and repletion is characterized by a hierarchical style, which means that Se is preferentially incorporated into some selenoproteins depending upon the significance of the physiological role of each particular selenoprotein. Therefore, some selenoproteins respond fast to the lack of dietary Se with a loss of activity; others remain stable when deficiency is moderate, and only decrease upon prolonged and substantial Se depletion (Brigelius-Flohe, 1999).

A dietary restriction of Se may affect a number of metabolic functions; however, the activity of selenoproteins in specific tissues (i.e. endocrine and nervous systems) may remain unchanged. For instance, under Se deprivation, the activity of selenoproteins in the liver, kidneys, and lungs decrease; whereas activities exhibited in the brain remain at a level similar to that during normal Se intake. Cytosolic GPx declines under Se restriction when most of selenoproteins are still near normal expression values (Brigelius-Flohe, 1999). Selenium is excreted from the body via milk, exhalation, urine, endogenous excretion and feces. The latter is the most important route of excretion in ruminants as a consequence of the extensive mineral reduction by ruminal microbes, which convert Se into insoluble selenides. The total fecal excretion may reach up to 68% of Se intake (Symonds et al., 1981b), where endogenous Se represents between 22% and 36% of the fecal excretion (Koenig et al., 1991a). The variation may be related to the amount of organic matter intake (OMI), as there is a general agreement that fecal Se excretion is more sensitive to OMI than to Se intake itself (Krishnamurti et al., 1997).

Urine is another important route for Se excretion, but it requires a metabolic transformation to trymethil selenide prior to urinary excretion (Krishnamurti et al., 1989). Kinetic trials have shown that urinary excretion ranges between 2.7 and 6.0% of Se intake in cattle (Symonds et al., 1981a; Symonds et al., 1981b), and 7% in ewes (Krishnamurti et al., 1989). Additionally, significant amounts of Se are excreted into the alimentary tract, where the possible routes of this endogenous loss are saliva, bile and other gastrointestinal secretions

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(Symonds et al., 1981b). The biliary excretion in cattle (approximately 0.2%) is less important (Symonds et al., 1981a), but it may be higher as the OMI increases (Krishnamurti et al., 1997). The total endogenous fecal loss has been estimated, on average, at 13% of Se intake, but the ratio of endogenous fecal Se to intake is significantly higher in Se deficient than in normal animals (Krishnamurti et al., 1989; Krishnamurti et al., 1997).

1.2.3 Transfer to Milk

Milk Se content is highly correlated with blood and serum Se concentrations; therefore, that relationship supports the use of milk as a diagnostic aid to monitor the Se status in individual lactating cows, and bulk tank milk Se concentration (BTSe) may be used to establish the Se status in lactating cows (Grace et al., 2001; Wichtel et al., 2004). When BTSe is ≤ 0.05 μmol/L, collecting blood samples from individual cows may be required to further assess the herd Se status (Grace et al., 2001). However, based on a regression model of the mean herd serum Se concentration on the BTSe concentration in 15 Prince Edward Island dairy herds with widely differing BTSe concentrations, a BTSe < 0.12 μmol/L represented deficiency, and a concentration > 0.28 μmol/L corresponded to Se adequacy (Wichtel et al., 2004).

While meeting Se requirements is important to achieve a balance between intake and excretion, supplementation can enhance the nutritional value of milk. Newborns, infants and elderly people older consumers may benefit from consuming Se-enriched milk (Knowles et al., 2006). Therefore, milk becomes a potential source to increase Se consumption in humans who are at risk of Se inadequacy.

Selenium easily diffuses into the mammary gland; therefore, the milk is another route of excretion. Selenite diffuses into the mammary gland after the hepatic reduction of the mineral, and most of dietary Se transferred to milk is associated with proteins (Allen and Miller, 1980; Knowles et al., 1999). The transfer of Se into milk occurs through passive diffusion, but some

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active transfer also occurs (Allen and Miller, 1980). Notwithstanding, the amount diffused into milk is affected by several factors, such as Se source and dose (Phipps et al., 2008). Selenite cannot bind to cow milk proteins (Aspila, 1991) or be converted directly to selenoamino acids for incorporation into casein (Allen and Miller, 1980). In contrast, Se coming from organic sources (i.e. Se-met) can be directly incorporated into milk protein, explained by a direct substitution of Se-containing amino acids for sulfur-containing amino acids (Allen and Miller, 1980; Phipps et al., 2008).

Only a small portion of Se is transferred into milk after using inorganic forms of Se (e.g. sodium selenite) (Conrad and Moxon, 1979). These authors indicated that selenite increased milk Se content when cows were fed rations low in naturally occurring Se, but a much more marked effect was found when cows were fed naturally-occurring Se. The authors concluded that naturally-occurring Se is transferred more readily into milk, probably due to its greater bioavailability. More recent studies have found that a higher amount of Se was transferred to milk when an organic Se source was fed (Knowles et al., 1999; Phipps et al., 2008).

A milk Se concentration between 0.13 and 0.32 μmol/L has been considered as adequate (NRC, 2001), but an increase in Se intake would not produce a substantial increase in milk Se content when cows are fed Se-adequate rations. A plateau in milk Se concentration is reached when the dietary intake varies between 6 and 11 mg/d (Maus et al., 1980; Aspila, 1991). That plateau is reached at a different dietary intake for inorganic and organic sources, suggesting that the level of saturation for Se excretion via milk is different for inorganic and organic sources of Se (Aspila, 1991). There is a large variation in the results of clinical trials to evaluate the effect of supplementation on milk Se concentration, where several sources and dose levels have been tested (e.g. Aspila, 1991; Knowles et al., 1999; Juniper et al., 2006). Therefore, to know what is the magnitude of the difference in milk Se concentration due to the dose of supplemental Se, either organic or inorganic remains as a matter of interest.

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Selenium supplementation also affects the Se content of the milk casein fraction. In a New Zealand study, the effect of supplementation on casein Se concentration mirrored that observed in whole milk, and approximately 71% of milk Se was found in the casein fraction (Knowles et al., 1999). Recent studies found a different milk Se distribution: 53.6, 42.6 and 9.3% were found in whey, casein and fat fractions, respectively; these being unaffected by the source of Se (Muniz-Naveiro et al., 2005).

1.3   Selenium Requirements

A nutritional requirement is the minimum absorbed amount of any nutrient required that will match the fecal excretion (including endogenous losses), retention in body tissues, and excretion in milk. The nutritional requirements of Se for beef and dairy cattle have been assessed by three classical methods:

i.   Empirical experiments (Oh et al., 1976);

ii.   Clinical response to supplementation trials (NRC, 2001; Underwood and Suttle, 2001);

iii.   Factorial calculation (Grace, 1992; Underwood and Suttle, 2001).

Most of the trials where Se supplementation has been associated with the reduction of disease prevalence have used a dietary Se concentration ranging from 0.35 to 0.40 mg/kg DM, which is slightly higher than the suggested amount necessary to reach a positive Se balance (NRC, 2001). When the diet Se concentration is 0.40 mg/kg DM, the daily Se intake will be higher than 6 mg/d if the cow consumes more than 15 kg DM/d, which is the level needed to reach a plateau in milk and plasma Se concentrations (Maus et al., 1980). Consequently, the Subcommittee on Dairy Cattle Nutrition of the National Research Council decided to set the Se

requirement for dairy cattle at 0.3 mg/kg DM (NRC, 2001). Clinical trials with beef cattle have reported beneficial results using less Se than trials in dairy cattle; therefore, the Subcommittee on Beef Cattle has set the Se requirement for beef cattle at 0.1 mg/kg DM (NRC, 2000). Although no new data have been added to these recommendations, the Se requirement of dairy cattle may be higher for an optimal immune function (Smith et al., 1984; Smith et al., 1985; NRC, 2001). Nevertheless, results from trials in New Zealand suggested that reproduction and immune function are not greatly impaired in dairy cattle moderately deficient in Se (Grace, 1992; Wichtel, 1998a).

The factorial calculation is another approach used to estimate Se requirements. The concept of the factorial calculation is that the dietary requirement is defined as the amount of nutrient needed to replace its utilization for each major biological function and loss (e.g. maintenance, endogenous loss, growth, fetal growth, and milk production). These values are added up to derive the daily net requirement, and then further adjusted by the coefficient of TA and dry matter intake (DMI) to estimate the concentration required in the diet (Grace, 1992). This methodology covers a wide production ranges, makes possible to identify the determinants of the requirement, and provides a basis for assessing the validity of extremes values for TA and fecal loss of Se (Underwood and Suttle, 2001). Nevertheless, the method may have limitations, as the deposition of Se in body tissues, transfer to fetus, and the amount excreted in milk vary with Se intake (NRC, 2001).

The approximate amounts taken up by conceptus, lost and excreted from the body, and excreted in milk are given in Table 1.3. The requirement of Se for a 600-kg cow producing 30 kg of milk/d, and ingesting 19 kg DM/d is given in Table 1.4.

There are substantial differences among the factorial estimation, the ARC and NRC recommendations, and the researchers from New Zealand. The endogenous loss and the amount excreted in milk are substantially lower than those values indicated by Grace (1992), and some

estimations for cattle have been extrapolated from sheep, which seem to be more susceptible than cattle to Se deficiency (Underwood and Suttle, 2001). Moreover, the factorial approach depends upon accurate values for endogenous loss and absorption, these being associated with the greatest errors (Grace, 1992). Clearly, all New Zealand dairy cows would be Se deficient, when this is not so. The low incidence of some of the Se-responsive disorders in New Zealand suggests that the performance of cattle moderately deficient in Se is not greatly impaired (Wichtel, 1998a). The NRC has adopted its recommendation for Se intake based on the results of observational and experimental studies highly variable in design, and, most of which were conducted in intensively managed herds where pasture was not the principal feed (Harrison and Conrad, 1984a; Harrison and Conrad, 1984b; Wichtel, 1998b). As an example, NRC (2001) indicates that blood Se concentration should be greater than 2.2 μmol/L as an acceptable cut-off point to optimize udder health (Jukola et al., 1996). Other studies have indicated that 1.3 μmol/L is an adequate concentration (Gerloff, 1992). These blood concentrations can be maintained at an intake of 6 mg/day of Se; after this point, the blood Se concentration response was limited (Maus et al., 1980). This intake is fairly close to the factorial calculation described in Table 1.4, and to NRC suggestion, but much more than the ARC (1980) and Grace (1992) calculations.

The discrepancy among the different approaches to Se requirements may arise from differences in other dietary factors, such as the content of other antioxidants or vitamin E, or to different oxidant challenges experienced by New Zealand and overseas cattle (Wichtel, 1998b; Underwood and Suttle, 2001). Such discrepancies may also be associated with different criteria to interpret the association between the results of field observations (i.e. prevalence of Se-responsive disorders) and Se supplementation levels among trials carried out in New Zealand, Australia, United Kingdom or the United States of America (ARC, 1980; Lee et al., 1999). There are also differences in the approach to Se requirements when they were set using the adequacy of Se balance measured by plasma or whole blood Se concentration (NRC, 2001).

Table 1.3. Estimates of selenium excreted in urine, endogenous loss, and requirement for liveweight gain, gestation, and milk yield.

	Selenium	Reference
Maintenance		
Urine excretion (µg/kg LW)	2.2	Symonds et al. (1981a)
Endogenous loss (µg/kg DMI)[1]	8.3 - 18.4	Koenig et al. (1991a), Koenig et al. (1991b)
Liveweight gain (µg/kg)	51	Grace (1992)
Gestation (µg/d)[2]	55	House and Bell (1994)
Milk yield (µg/kg)[3]	16	NRC (2001)

[1]Variation depends upon DMI.
[2]Requirement for the last trimester of gestation, where d corresponds to days of gestation.
[3]Estimated as 6 µg/kg for New Zealand cattle (Grace, 1992).

Table 1.4. Comparison of estimated dietary selenium requirements (mg/d) and dietary selenium concentration (mg/kg DM) for a 600-kg dairy cow producing 30 kg of milk/d, and consuming 19 kg DM/d.[1]

	Requirement (mg/d)			
	Factorial calculation	Grace (1992)	ARC (1980)	NRC (2001)
Maintenance		0.15		
Urine excretion	1.30			1.00
Endogenous loss	0.16			0.38
Milk yield	0.48	0.18		0.75
Net requirement	1.94	0.33		2.13
Gross requirement[2]	4.85	0.83	1.9	5.33
Diet concentration (mg/kg DM)	0.26	0.04	0.10	0.30

[1]Estimated by a regression equation (NRC, 2001).
[2]Estimated as dietary basis allowing the fact that about 40% of Se is absorbed (Weiss, 2005). If extremely low values were representative, estimated gross requirement would become impossibly high.

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The adoption of the NRC recommendation to adjust the Se intake in pasture-based cattle remains controversial and requires more investigation to clarify the guidelines for supplementation. It should be noted that New Zealand trials carried out to evaluate the response to Se supplementation have been similar experimental designs, whereas overseas trials are variable in design, specially with regard to sources and dose rates of Se (Wichtel, 1998b).

Selenium was approved as a feed additive for dairy cattle diets in 1979 at an added level of 0.1 mg/kg DM, and increased to 0.3 mg/kg DM in April 1987, which is the current recommendation (Gerloff, 1992; Ullrey, 1992; NRC, 2001). The maximum tolerable level of dietary Se for cattle has been set at 5.0 mg/kg DM, which is about 17 times its requirement (NRC, 2005).

1.4   Selenium Supplements and Methods of Supplementation

Selenoamino acids provided by basal feedstuffs, inorganic Se (i.e. sodium selenate or selenite), and organic Se (i.e. Se yeast) are the most common forms of oral Se supplementation for ruminants. However, other inorganic forms (e.g. selenium dioxide, barium selenate, etc), and methods of supplementing (i.e. parenteral) Se are available (Grace et al., 1995).

Intra-ruminal Se pellets (Wichtel et al., 1994), and long-acting Se forms (Mallinson et al., 1985) have been shown to be effective methods for supplementing Se. The slow-release Se form corresponds to a unique formulation of barium selenate that acts as a depot under the skin, and protects against Se deficiency for at least 12 months, releasing approximately 0.8 mg Se/d (Mallinson et al., 1985; Grace et al., 2001). Long-acting products are widely used outside of North America, and are useful for supplementation in dairy or beef systems where cattle spend a significant proportion of the year on pasture.

Organic forms, such as Se yeast, are obtained by yeast cultivation using a fed-batch fermentation process that provides incremental amounts of Se salts, minimizing the detrimental effects of Se salts on the yeast. This allows for the optimal incorporation of inorganic Se into cellular organic material, acquiring similar properties to natural Se sources (Weiss, 2005). Organic Se yeast contains a variety of Se proteins, mainly Se-met, and other low molecular weight seleno-compounds, and has probably been the most widely investigated natural product containing Se (Połatajko et al., 2004).

The amount of Se to be supplemented, and the frequency of administration have been determined after considering the requirements, and the efficacy of the various Se supplements. Any plan for Se supplementation requires a thorough analysis of the animal characteristics, production records, and feedstuff micronutrient content to ensure that regulatory requirements are met. The methods for supplementation, dose rates suggested, and the effective duration for the most common available Se supplements are summarized in Table 1.5.

1.5   Assessment of Selenium Status

The aim of Se status evaluation is to establish whether the animal is being adequately nourished with Se. This assessment acquired a different dimension after the discovery of Se essentiality in the 1950s, there has been a permanent interest of dairy producers and advisers for using serum Se assays as an integral part of dairy cattle population medicine since then (Stowe and Herdt, 1992).

Table 1.5. Method of supplementation, source, dose rate and duration of selenium supplementation for adult cattle.[1]

Method	Source	Dose rate		Duration (months)
Discontinous				
Oral drench	Sodium selenate	0.05	mg/kg LW	1
Parenteral injection	Barium selenate	1		12
	Sodium selenate	0.1		1.5
	Sodium selenite	0.05 - 0.10		2 - 3
Intra-ruminal pellet	Elemental Se (10%)	6	g	12
. Continous[2]				
Drench	Sodium selenate	1 - 8	mg/d	
Mineral mixes[3]	Sodium selenate/selenite	1 - 8		
	Se yeast[4]	0.10 - 0.15	mg/kg DM	
Indirect				
Fertilizers	Sodium selenate	5 - 10	g/Ha	12
	Selenium granules[5]	5 - 10		24[6]

[1]Adapted from: Mallinson et al. (1985); Wichtel et al. (1994); Wichtel (1998b); Hartikainen and Ekholm (2001); Underwood and Suttle (2001).
[2]Selenium is consumed on a daily basis.
[3]A controlled analysis of basal diet is required when using free-choice mineral mixes.
[4]Used as feed additive, mg/kg of complete feedstuff.
[5]Selenium as sodium and barium selenate (90%), and sodium and barium selenite (10%).
[6]Provides up to 24 months of supplemental Se for livestock when used at 10 g/Ha.

Selenium status has been evaluated by measuring its concentration in samples that can be easily obtained from the animal (Wichtel, 1998b). Whole blood, plasma, serum, and milk Se concentration, and plasma, platelet and whole blood GPx activity have been commonly used to establish Se status (Ullrey, 1987; Wichtel, 1998b; Ashton et al., 2009). There are measurable changes in those compartments before clinical signs of deficiency are evident.

The consideration of blood GPx activity as a biomarker for Se status started with two concurrent discoveries in 1970s. The enzyme was classified as a selenoprotein containing a large portion of erythrocyte Se (Rotruck et al., 1973), while other study found that GPx contained 4 g atoms Se/mole in its active site (Flohe et al., 1973), in particular as Se-cys (Burk, 1991). Further, the distribution of blood GPx in cattle was examined, finding that 98% of its activity was associated with erythrocytes, and that blood cells comprised ~ 73% of whole blood Se (Scholz and Hutchinson, 1979). Therefore, blood GPx evaluation has been considered a good functional index of dietary Se intake (Anderson et al., 1978; Thompson et al., 1981; Grace et al., 2001).

Although the evaluation of blood GPx activity has been used as a useful biomarker of Se status, the early popularity of this analysis for veterinary clinical purposes has waned due to certain weaknesses to accurately establish Se status (Stowe and Herdt, 1992). There are a number of important reasons that arise from the literature to explain this. First, whole blood GPx activity has much laboratory-to-laboratory variation due to the use of different analytical techniques, and units used to express activity. This precludes setting applicable standards for establishing optimal GPx activity, and the limits of normalcy established in one laboratory will not be applicable in others (Ullrey, 1987; Stowe and Herdt, 1992).

Second, there is a delay between increasing Se intake and the rise in whole blood GPx activity. The incorporation of Se into the enzyme structure occurs during de novo synthesis (i.e. erythropoiesis) rather than adding Se to an apoenzyme (Knowles et al., 1999). Due to the slow nature of that process, a lag (~ 45 d) occurs before blood GPx activity catches up with the

increase of Se concentration in other compartments, such as plasma or serum (Thompson et al., 1981; Grace et al., 2001). Therefore, a minimum of 30 days lag time between the beginning of supplementation and the evaluation of blood GPx activity is needed to identify a change in Se. A recent meta-analysis found that the duration of the trials using blood GPx as indicator of Se status ranged between 17 and 20 wk, suggesting that blood GPx is a biomarker of Se status to be used in long-term studies (Ashton et al., 2009).

A third factor is related to the plateau in blood GPx activity, after which the relationship between Se intake and blood GPx is no longer linear. A well-established plateau of blood GPx is reached in ruminants after Se supplementation (Oh et al., 1976; Wolf, 1998), and in non-ruminants (Rea et al., 1979; Sunde et al., 2005). After blood GPx reaches a plateau or if Se status is considered adequate, the relationship between blood Se and blood GPx activity is poor (Ullrey, 1987), limiting the use of blood GPX as a tool for establishing Se status under conditions of Se adequacy.

The chemical form of the supplement can also influence the response of blood GPx to Se supplementation. Studies conducted with non-ruminants have found a higher concentration of Se in erythrocytes when Se-met was supplemented (Butler et al., 1990). However, the amount of erythrocyte Se associated with GPx was higher in animals supplemented with sodium selenite, indicating that the chemical form has a significant influence on Se distribution among erythrocyte proteins, such as GPx (Butler et al., 1990).

1.6   Selenium and Udder Immune Response

The most convincing evidence about the influence of Se on the immune response and disease resistance relates to its role in mastitis control. Investigators from Ohio State University

first established a beneficial effect of Se supplementation on udder health in early 1980s (Smith et al., 1984; Smith et al., 1985). Selenium supplementation, as single effect, did not affect the incidence of CM, but the duration of CM cases was shorter in Se-supplemented cows than in unsupplemented controls. Environmental pathogens (i.e. coliforms and streptococci different than *Strep. agalactiae*) were the most frequent mammary pathogens isolated (Smith et al., 1984). Further, Smith et al. (1985) found that Se supplementation in intensively managed heifers was highly protective at calving, reducing by 42% the prevalence of IMI caused by staphylococci different than *Staph. aureus*. In addition, there were fewer cases of CM, the duration of cases was shorter, and there were lower SCC in the milk of Se-supplemented primiparous heifers compared to unsupplemented controls.

1.6.1 Antioxidants and Mastitis

Mastitis, or inflammation of the mammary gland, remains the most costly disease in dairy cows; economical losses are due to decreased milk yield, costs of drugs and treatment, early culling, extra labor, discarded milk, and increased rate of cow replacement (Halasa et al., 2007). Subclinical or clinical mastitis (CM) is the result of a disruption in the relationship of three major components of the epidemiologic triad: presence of pathogens, host resistance, and the environment (Bradley, 2002).

Suboptimal nutrition is a recognized risk factor for an increased susceptibility to infectious diseases in cattle (e.g. mastitis), and the role of micronutrients in specific and non-specific immune udder defenses have been reviewed (Smith et al., 1997; Hemingway, 1999; Burton et al., 2000; Sordillo and Aitken, 2009). The supplementation with specific micronutrients (e.g. selenium) and its effect on udder health has been most extensively studied in the context of its role in mastitis and its control (Smith et al., 1984; Hogan et al., 1990; Malbe et

al., 2003). However, no beneficial effect of Se supplementation on udder health has been found (Wichtel et al., 1994; Grace et al., 1997; Weiss and Hogan, 2005).

The biological action of Se for enhancing the immune response of the mammary gland is thought to be mediated through the expression of selenoproteins that have antioxidant properties (Bruzelius et al., 2007; Sordillo et al., 2007; Aitken et al., 2009). The polymorphonuclear cells (PMN) release noxious molecules when they reach infected sites (e.g. the mammary gland following an IMI), which are needed to kill the pathogens involved in the process. These molecules, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, come from the respiratory burst and are injurious to the mammary epithelial cells due to their action as oxidants (Ledbetter et al., 2001; Halliwell, 2006; Sordillo and Aitken, 2009). The cytotoxic effects result from oxidation of proteins, amino acids, and DNA. Therefore, the use of different antioxidants or free radical scavengers may be effective for protecting mammary epithelial cells against neutrophil-induced oxidative stress (Bruzelius et al., 2007; Sordillo et al., 2007; Sordillo and Aitken, 2009). Deficiencies in antioxidants, the natural protection against oxidants, impair the equilibrium with oxidants, resulting in oxidative stress that affects body tissues (Miller et al., 1993; Halliwell, 2006).

Early studies with ruminants have suggested that humoral immune responses are enhanced after Se and vitamin E supplementation (Stabel et al., 1989; Nemec et al., 1990). These studies indicated an increase in the production of immunoglobulins after exposure to different pathogens or vaccines. However, whether Se supplementation affects the humoral response of the udder after an infection is established or after vaccination is unknown.

Supplementation with Se and other antioxidants, such as vitamin E, enhances leukocyte function in the udder (Hogan et al., 1990). Recent studies have suggested that particular nutrients (e.g. antioxidants) modify gene expression in blood and milk leukocytes (Burton et al., 2000;

Sordillo et al., 2007). Thus, the effect of Se inadequacy on bovine immune responsiveness includes:

    i.   Decreased expression of selenoproteins, such as GPx and TrxR (Bruzelius et al., 2007; Sordillo et al., 2007);

    ii.   Reduced production of chemoattractants (Ndiweni and Finch, 1996; Arthur et al., 2003);

    iii.   Altered metabolism of arachidonate, which affects the production of eicosanoids or inflammation mediators (Cao et al., 1992);

    iv.   Decreased bactericidal capacity of bovine neutrophils (Gyang et al., 1984);

    v.   Damage to the epithelial cells of the mammary gland through the release of oxidant molecules by activated neutrophils (Lauzon et al., 2005);

    vi.   Inhibited lymphocyte proliferation (Cao et al., 1992).

However, there is a poor understanding of the exact mechanisms of action of Se and vitamin E on altered cellular function. Thus, a better understanding of the link between antioxidants and leukocyte biology at calving may help to develop effective mastitis prevention strategies.

1.6.2 *Mechanism of Action of Selenium on the Immune Response of the Mammary Gland*

In the early 1980s, studies on the phagocytic activity of PMNs demonstrated that phagocytosis was not affected in neutrophils isolated from Se-deficient heifers, while their ability to kill phagocytised pathogens was decreased (Boyne and Arthur, 1981; Grasso et al., 1990). This effect was further evaluated, and it seems that Se affects nonspecific immune indices, humoral immunity, cell-mediated immunity and cytotoxicity (Arthur et al., 2003). In addition, Se, via selenoproteins, may influence three areas of cell metabolism and function:

i.   Reduced activity of myeloperoxidase and NADPH-oxidase, limiting the mechanism of the oxidative burst (Erskine et al., 1989);

ii.   The ability of macrophages to detoxify the free radicals generated after phagocytosis (Gyang et al., 1984);

iii.   Reduction in the catabolism of hydrogen peroxide (Grasso et al., 1990);

iv.   Alterations in the metabolism of thyroid hormones and thymic cells (Arthur et al., 2003).

The loss of any of these functions would result in less efficient neutrophil bactericidal activity in vitro (Ndiweni and Finch, 1996) or in vivo (Hogan et al., 1990). Consequently, an impaired antioxidant defense leads to a reduction in the catabolism of oxidant molecules, affecting the mammary gland with a loss of secretory tissue where neutrophils have been accumulated (i.e. after an IMI), and the reduction of milk yield in those cows having an affected gland (Grasso et al., 1990). However, the effect of cytosolic antioxidants (i.e. GPx) seems to be paradoxical. Free radicals produced during the respiratory burst after phagocytosis can kill the neutrophils themselves, and the ability to continue to produce radicals depends upon Se status and neutrophil GPx activity. At the same time, an increased activity of GPx is required to protect the neutrophils themselves from the radicals (Erskine et al., 1989; Arthur et al., 2003). Therefore, there is more than one Se-dependent function that regulates the ability of immune cells to kill ingested pathogens.

In essence, antioxidant micronutrients, such as Se, reduce or scavenge oxidants that are produced during the respiratory burst, protecting leukocytes from killing themselves. Such attenuation of inflammation likely would explain why IMI are less severe and eliminated more quickly in Se-replete animals.

1.6.3 *Selenium Supplementation and Mastitis*

The association between oxidation and mastitis, and the mechanism of action of Se on the immune response of the mammary gland has been described. Several observational studies (Erskine et al., 1987; Weiss et al., 1990), and both in vitro (Ndiweni and Finch, 1996) and in vivo (Smith et al., 1984; Smith et al., 1985; Malbe et al., 1995) have found that Se supplementation is associated with lower prevalence, incidence, and severity of IMI and CM, and lower SCC in individual cows and bulk tank milk.

The incidence of CM has been reduced 37% in cows injected with Se (0.1 mg/kg LW) before calving compared to unsupplemented cows (Smith et al., 1984). Further, heifers fed with 2 µg Se (sodium selenite)/kg LW/d, and injected subcutaneously with Se (0.1 mg/kg LW) and vitamin E three weeks before calving, had a reduction of 42% in IMI prevalence and CM incidence around calving (Smith et al., 1985). Further, a negative relationship between the incidence of CM caused by environmental pathogens and plasma Se concentration was found in dairy cows from herds that had good control of CM caused by *Streptococcus agalactiae* and *Staphylococcus aureus* (Weiss et al., 1990).

Several trials have been performed using organic Se sources, but the outcomes of interest have been plasma or blood Se concentration, GPx activity (Ortman and Pehrson, 1997), and milk Se concentration (Knowles et al., 1999; Ortman and Pehrson, 1999). As to the immune response of the udder, non-infected quarters of cows supplemented with Se yeast were nearly 10 times more likely to be pathogen-free after 8 weeks when compared to quarters from unsupplemented cows (Malbe et al., 2003). However, the cure rate for infected quarters was not different between supplemented and unsupplemented cows.

In contrast to the published evidence since the early 1980s, there are a number of studies that found a non-significant effect of Se supplementation on udder health (Coe et al., 1993;

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Weiss and Hogan, 2005; Bourne et al., 2008; Nyman et al., 2008). The lack of a treatment effect on udder immune responsiveness might be explained by several factors:

i.   Selenium and/or vitamin E status before treatment;

ii.   The source of supplemental Se might have confounded the interpretation of results;

iii. Effects might have been masked by other nutritional factors not considered;

iv.   The additional management practices involving sanitation, periparturient care, or milking procedures might have been differently conducted among studies; or

v.   Insufficient statistical power in some of the studies.

1.6.4   Selenium Supplementation and Somatic Cell Count

Quarter- and cow-level SCC represent the inflammatory status of the mammary gland, and they reflect the udder health and the quality of raw milk, thus the milk from uninfected quarters have a mean SCC of approximately 70,000 cells/mL (Dohoo and Leslie, 1991; Schukken et al., 2003).

Parameters derived from SCC are often used to distinguish between infected and uninfected quarters. A cut-off between 200,000 and 250,000 cells/mL has been set as optimal to reduce diagnostic error (Dohoo and Leslie, 1991). These values are not considered a physiological cell content in milk distinguishing healthy from unhealthy quarters, but it has been considered as a practical approach under field conditions (Schukken et al., 2003).

The mean SCC of uninfected quarters increases with age, and days in milk. However, the quarter-level infection status was the main factor to explain SCC variations (Schepers et al., 1997). Cell counts increase quickly after an infection takes place in the udder, when a massive influx of PMN cells into milk is observed. After the infection is eliminated, SCC returns to normal within a few weeks. When the immune system is unable to remove bacteria, a chronic infection results in a continuous trigger, and SCC is high for long time (Schukken et al., 2003).

In the United States of America, herds with high SCC (> 700,000 cells/mL) had lower

blood GPx activity than did herds with lower SCC (~ 150,000 cells/mL) (Erskine et al., 1987).

Observational studies carried out in England found a negative correlation between bulk tank

SCC and the blood GPx activity (Ndiweni et al., 1991). A recent trial conducted in Southern

Chile found that mean natural logarithm of SCC (LnSCC) was lower in Se-supplemented cows

than in unsupplemented controls after an intramammary challenge with *Staph. aureus* at 140

days in milk (Kruze et al., 2007). These results suggest a beneficial effect of Se on the udder

immune response.

There are also observational studies that found no association between Se status and

SCC in milk (Grace et al., 1997; Kommisrud et al., 2005). Clinical trials on Se supplementation

have indicated that supplementation did not affect SCC in treated cows compared to the

unsupplemented controls (Whelan et al., 1992; Coe et al., 1993; Wichtel et al., 1994).

1.7   Objective

Based on the current evidence where contradictory results are still found, and the

discrepancy between Se requirements for pasture-based and intensively managed herds, more

investigation is required to establish clear guidelines for Se supplementation when the objective

is to improve the health status of the mammary gland. Whether the typically higher Se status (as

measured by a higher blood GPx activity) when cows are injected with barium selenate or fed Se

yeast reflects improved udder health compared to Se-supplemented cows with traditional

inorganic sources (e.g. sodium selenite) is unknown. In addition, clear guidelines on optimal Se

supplementation practices for grazing heifers, in particular to enhance the udder immune

response, are not available to herd managers at present. Moreover, the effect of injectable

sustained-release inorganic Se products on udder immunity has not been adequately investigated to date, perhaps because such products are not yet licensed for use in North America.

The overall hypothesis for this thesis is that an adequate Se status or supplementation with injectable barium selenate or oral Se yeast may be associated with an improvement of measures of udder health (e.g. lower risk of new intramammary infections, and reduced SCC). Thus, the aim of this thesis was to evaluate the effect of Se status and Se supplementation on milk Se concentration, and on indicators of udder health.

1.7.1   Specific objectives

The main objective was split into five parts:

i.   To summarize, through a systematic review and a meta-analysis, all available scientific evidence related to the effect of oral Se supplementation on milk Se concentration in cattle (Chapter 2).

ii.   To determine the association of milk Se concentration with SCC and the risk of IMI around calving in dairy cows from Atlantic Canadian dairy herds (Chapter 3).

iii.   To evaluate the association between bulk tank milk Se concentration with milk production parameters, bulk tank SCC, and the prevalence of *Staph. aureus* in bulk tank samples in Canadian dairy herds (Chapter 4).

iv.   To determine the effect of a single prepartum Se injection, using barium selenate as the Se source, on both incidence risk and incidence rate of overall and pathogen-specific new IMI, and on SCC during lactation in pasture-based dairy cows (Chapter 5).

v.   To evaluate the effect of Se supplementation using barium selenate or Se yeast

before calving on the risk of IMI, and SCC around calving in first-lactation

dairy heifers raised in pastoral systems (Chapter 6).

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2   META-ANALYSIS OF THE EFFECT OF ORAL SELENIUM

SUPPLEMENTATION ON MILK SELENIUM CONCENTRATION IN CATTLE[1]

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[1] Ceballos, A., J. Sanchez, H. Stryhn, J. B. Montgomery, H. W. Barkema, and J. J. Wichtel. 2009. Meta-analysis of the effect of oral selenium supplementation on milk selenium concentration in cattle. J. Dairy Sci. 92:324-342.

2.1 Abstract

Soils in many regions of the world have low selenium (Se) content. Consequently, forages and crops grown on these soils may provide inadequate dietary Se for grazing animals and humans. Se supplementation has been used to enhance Se status and milk Se concentration, but results have conflicted. Milk Se concentration appears to be a useful indicator of animal and herd Se status, and reflects the response to supplementation. A systematic review and meta-analysis were carried out to summarize all available scientific evidence for the effect of oral Se supplementation on milk Se concentration in cattle. The literature search was based on electronic and non-electronic databases. Fixed- and random-effects models of meta-analysis were used, and a meta-regression was carried out to evaluate heterogeneity among studies. Random-effects meta-analysis was performed on 42 studies published between 1977 and 2007. Oral Se supplementation resulted in an average increase in milk Se content of 0.16 (95% CI: 0.12, 0.21) $\mu mol/L$ ($P < 0.05$), with a significant heterogeneity among studies. Weak publication bias was evident, but it did not change the average effect. The continent where the study was performed, Se source, Se dose and the interaction between source and dose explained 71% of the between-study variance. On average, American cows supplemented with Se yeast (e.g. 6 mg/cow/d) had higher milk Se concentration (approximately 0.37 $\mu mol/L$) 75 days after the beginning of supplementation when compared to those supplemented with inorganic forms of Se. This information provides a basis for tailoring daily animal requirements and a means of enhancing Se intake in consumers of dairy products.

2.2 Introduction

Selenium is a naturally occurring solid substance typically defined as non-metallic, occurring worldwide but distributed unevenly in soils (Oldfield, 2002). Many regions of the world have soils with low Se content; as a consequence, feedstuffs grown on these soils may provide inadequate dietary Se for grazing animals and humans. Selenium nutritional requirements for beef and dairy cattle have been set at 0.1 and 0.3 mg/kg on a DM basis, respectively (NRC 2000, 2001). Though Se is not an essential nutrient for plant growth, some agricultural practices (e.g. fertilization) may contribute to reduce the content of Se in soils and plants (Hartikainen, 2005). The consumption of animal products derived from animals grazing in low-Se areas can influence the Se status of entire human communities, putting humans at risk of overt deficiency (WHO/FAO, 2004). Some countries have recognized this risk; for instance, in Finland, where Se deficiency is known to exits, Se fertilization is required for grain crops destined to human consumption (Hartikainen and Ekholm, 2001).

The daily consumption of 100 g of milk will provide at least 10% of the daily Se requirement for adults (Knowles et al., 2004). Moreover, milk-based formulas used for infants should provide at least 10 µg Se/day to complement the maternal supply (WHO/FAO, 2004), but non-fortified cow milk-based formulas will often not provide this amount (Carver, 2003). Consequently, tailoring dairy products to meet specific requirements of a population, such as enhanced Se intake, is an attractive concept for the promotion of human health (Knowles et al., 1999; Knowles, et al., 2004).

Feeding systems designed to increase milk Se content have been developed (Knowles, et al., 1999; Grace et al., 2001; Guyot et al., 2007). Early studies in the 1970s indicated that a relatively small proportion of Se was transferred into milk after feeding inorganic forms of Se such as sodium selenite. Supplementation with sodium selenite increased milk Se content when

cows were fed rations low in naturally occurring Se, but milk Se concentration increase was lower compared to Se-supplemented cows that were fed with high naturally-occurring Se diets (Conrad and Moxon, 1979). In fact, many experiments have shown that Se supplementation results in an increase in Se content of milk, but it does not appear to increase linearly as Se intake increases (Conrad and Moxon, 1979). These authors concluded that Se from natural sources might be transferred more readily to milk, probably due to its greater bioavailability. In addition, an increase in Se intake would not produce important increases in milk Se content when cows are fed Se-adequate rations (Aspila, 1991). Further studies have reported different effects of Se supplementation on milk Se concentration, depending upon previous and current dietary Se content, source and route of administration (Aspila, 1991; Malbe et al., 1995; Knowles, et al., 1999), but not all attempts to increase milk Se concentration have been successful (Stowe et al., 1988; Gierus et al., 2003). Trials in cattle have shown variable results after using different sources, doses and routes of administration of Se, describing either no significant effects (Ammerman et al., 1980) or increased milk Se concentrations by as much as sevenfold (Guyot, et al., 2007).

The incorporation of Se into the various fractions of milk may vary depending on source and route of administration of the supplement. While this may have practical implications for the dairy processing industry, this has not been adequately examined to date. In addition, many supplementation trials have been conducted only for a short period of time, not being sufficient to reach a steady-state milk Se concentration after supplementation. Consequently, there is a need to summarize the response in cattle to different sources of supplementary Se. Clear guidelines as to how Se supplements should be administered to cattle, in particular to produce Se-fortified milk for human consumption currently are not available.

Narrative reviews have indicated a beneficial effect of Se supplementation on milk Se concentration (Conrad and Moxon, 1980; Weiss, 2005). Traditional narrative reviews have been

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widely used in veterinary literature to collate existing evidence on a particular intervention, but the majority of them do not use either a systematic or statistical method to identify, assess, and synthesize the information they are gathering. Narrative reviews are subjective, and based on preconceived opinions of the reviewer, therefore prone to bias (Sargeant et al., 2006). On the other hand, systematic reviews appraise critically, summarize and attempt to reconcile all published evidence concerning to a particular intervention (Jadad et al., 1997). They minimize systematic and random errors, and may or may not include a quantitative statistical analysis (meta-analysis) of the results of two or more studies to produce an average estimate of the treatment effect (Jadad et al., 1998; Sargeant, et al., 2006).

We hypothesized that milk Se concentration may be increased, reaching a biologically significant level for human consumption, and that using Se sources other than inorganic Se may cause a higher milk Se concentration. The objective of this study was to summarize, through a systematic review and a meta-analysis, all available scientific evidence related to the effect of oral Se supplementation on milk Se concentration in cattle.

2.3   Material and Methods

2.3.1   Literature Search

An electronic and non-electronic literature search was conducted to identify primary studies carried out between January of 1970 and March of 2008. The server of the University of Prince Edward Island was used to cover AGRICOLA (via CSA Illumina), CAB abstracts (via OvidSP), MEDLINE (via EBSCOhost), PubMed (via Internet), Science Direct, Web of Science, and WorldCat Basic Search. The keyword combinations were (ruminant OR bovine OR cattle OR cow* OR heifer*) AND (selenium OR glutathione peroxidase OR gsh-px OR gpx) AND

(experiment* OR trial* OR effect* OR study OR studies OR supplement*) AND (colostrum OR milk OR dairy products*). Primary studies published in English, French, Italian, Portuguese, Spanish and German, were included. There was no restriction to peer-reviewed journals, and the eligible publications included abstracts, conference proceedings, book chapters and theses. Additionally, the following proceedings were scanned for references: American Dairy Science Association (1970 to 2007) and American Society of Animal Science (1970 to 2007). All references related to milk Se concentration cited in three recent review papers (Weiss, 2005; Gierus, 2007; Guyot and Rollin, 2007) were also identified. In addition, different groups of investigators from the National Chung Hsing University (China), Primary Industries Research Victoria (Australia), Ohio State University (USA), Swedish University of Agricultural Sciences (Sweden), Università Cattolica del Sacro Cuore (Italy), and University of Veterinary and Pharmaceutical Sciences (Czech Republic) were contacted by email asking for unpublished studies related to the intervention of interest, and finally, the potential studies were combined with a set of studies recovered from the trial database of Alltech Inc (Nicholasville, KY, USA).

Manuscripts were excluded if the title or abstract indicated that the study pertained to species different than cattle, or pertained to supplementation trials enrolling animals other than first-calving heifers or multiparous cows, or if the milk Se concentration was not evaluated. Additionally, studies were excluded if cows were Se supplemented with sources other than sodium selenite, sodium selenate or Se yeast, since those forms are the most widely used for oral supplementation in cattle (Weiss, 2005).

2.3.2   Outcome Evaluated and Data Extraction

The mean difference in milk Se concentration between Se-supplemented and unsupplemented cows was the outcome of interest. The milk Se concentration increased sharply within the first 28 days of supplementation, and rapidly decreased when supplementation was

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discontinued. As only very few studies extended the supplementation beyond 170 days, only milk Se concentration data between 28 and 170 days were considered for the meta-analysis.

All results were transformed to µmol/L if the paper cited the results using different units. For unit standardization, 78.96 g/mol was used for Se molecular weight (Barthelmy, 2005), and, on average, 1,030 g/L for milk density (Goff, 2008). Clinical trials were included regardless of whether they were conducted in a randomized fashion.

The precision of the estimate was based on its reported standard error (SE) or on a SE calculated from standard deviations (SD) of treatment and control groups. In studies involving repeated measures on the same cows, estimates of milk Se concentration at different time points were computed, and the variance adjusted by an inflation factor given by the formula (Dohoo et al., 2009):

$$\sigma_{\bar{y}}^2 = \frac{\sigma_y^2}{m}[1 + (m - 1)\rho]$$

where σ_y^2 is the variance at each time point, m is the number of time points, ρ is the intraclass correlation coefficient (ICC) within cows, and $[1 + (m - 1)\rho]$ corresponds to the variance inflation factor (VIF). Different ICC values (i.e. 0.90, 0.75 and 0.25) were assumed to adjust for clustering within cows.

The same value for the SE or SD was used in both groups when the paper reported a common value for the study groups. A computation of a common SD was made reconstructing the statistical analysis, if the information needed was available. For example, if the paper only reported the mean for milk Se concentration and a P-value, possibly in the form $P < 0.05$, the SD was reconstructed under the assumption of a normal distribution analysis, as follows:

$$SD = (\bar{x}_2 - \bar{x}_1) / t_{(\alpha, df)} \sqrt{\frac{1}{n_2} + \frac{1}{n_1}}$$

where $\bar{x}_2 - \bar{x}_1$ represents the difference between means; $t_{(\alpha, \, d/E)}$ is the percentile from the reference distribution; and n is the sample size of each group. When an exact calculation of SD was not possible, a SD was imputed as the pooled SD (SD_p) from all the other available studies included in the meta-analysis (Furukawa et al., 2006).

In one manuscript the mean milk Se concentration and its SE were reported on log scale (Hidiroglou et al., 1987a). Assuming that log transformed values followed a normal distribution with the SD derived from the SE and sample size, values on log scale were recalculated by simulation, and then backtransformed to calculate the arithmetic mean and its standard error.

Additional considerations in the data extraction process were as follows. If a study contributed more than one set of observations because of reporting data separately by parity, or by study year, or by Se source or Se dose, data for each set of observations were recorded separately. Other information was recorded, if available, from the selected studies (Table 2.1). Two independent investigators extracted the information using a structured data-collection form, and the first author resolved the discrepancies after re-reviewing the paper.

2.3.3   Meta-Analysis

The effect of Se supplementation on milk Se concentration in cattle was evaluated carrying out fixed and random-effects meta-analyses. The results reported in this paper corresponded to the random-effects meta-analysis given the observed heterogeneity of the results across the studies. The Q and I^2 statistics were used to evaluate if heterogeneity was present in this study (Deeks et al., 2001; Higgins et al., 2003). The random-effects meta-analysis was estimated according to:

$$T_i = \theta + u_i + \varepsilon_i$$

$$Var\ (T_i) = \tau^2 + v_i$$

where T_i is the effect of the ith study; θ is the true effect, u_i is the random effect of study i^{th}; and ε_i

is the residual error. The variance of T_i was computed as the sum of the between-study variance (τ^2), and the within study variance (v_i) (Sutton et al., 2000). The random-effects meta-analysis was carried out via the method-of-moments estimation (DerSimonian and Laird, 1986). The result of the meta-analysis was presented graphically using a forest plot. Moreover, the prediction interval for the treatment effect of a new trial was also calculated and presented as part of the forest plot (Harris et al., 2008). This interval is a prediction of the range within which the milk Se concentration will lie in a new trial evaluating the effect of oral Se supplementation.

2.3.4 Publication Bias

Studies showing no effect, not written in English, or containing unfavorable results to the study sponsor, might be less likely to be published or included in the analysis than those reporting significant favorable results. This is known as publication bias (Sterne et al., 2001). Statistical (Begg's and Egger's tests) and graphical methods (funnel plot) were used to evaluate possible publication bias. Additionally, the "Trim and Fill" method was used to estimate and correct for an eventual publication bias. Studies having a large SE or low statistical effects (i.e. "small studies") were omitted (trimming) until a funnel plot became symmetrical. Further the "true" center of the plot was re-estimated, and then the omitted studies were replaced with their "missing" counterpart studies around the center (Duval and Tweedie, 2000; Sterne, et al., 2001). This method evaluates how much the average estimate of treatment effect changes if there are studies missing due to publication bias (Duval and Tweedie, 2000).

2.3.5 Meta-Regression

The meta-regression analysis is a regression-type analysis where each study is weighted by its precision It is an extension of the random-effects meta-analysis to estimate the extent to which one or more covariates explain heterogeneity in the treatment effects. The meta-regression

of the factors related to the quality and design of the study (Table 2.1) on the factor of interest was performed using the method-of-moments estimation (Sutton et al., 2000). This method of estimation was preferred for consistency with the other analyses. Other methods of estimation resulted in changes in the between-study variance, but had only minor impact on regression coefficients.

Unconditional analyses of continent where the study was performed, study population, parity, type of cattle production (e.g. dairy or beef), source of Se and dose of Se, days, stage of lactation, and frequency of administration were evaluated, and then unconditionally significant associated variables at $P < 0.15$ were retained to build a multivariable regression model. Further, the multivariable model was manually reduced by backward selection of the significant variables at $P < 0.05$. Each covariate was evaluated to determine, for each predictor, how much of the between-study variance was accounted for.

2.3.6   Cumulative Meta-Analysis

Cumulative meta-analysis is the product of performing a new meta-analysis every time a new trial is added to a series of trials. Repeated poolings, instead of a single pooling estimation, are performed as each study is added (Lau et al., 1995). Cumulative meta-analysis was used as an exploratory tool to identify retrospectively whenever the Se supplementation effect first reached statistical significance. Moreover, cumulative meta-analysis was used to correlate the accruing evidence with recommendations made by experts (Egger et al., 2001), and to identify eventual temporal patterns in the trial results (Lau et al., 1995).

Table 2.1. Additional information extracted from selected studies to evaluate the effect of oral Se supplementation on milk Se concentration in cattle.

Variable	Description
Quality of the study	
Publication type	Peer-reviewed journal, book chapter, abstract, proceedings, thesis, unpublished, other
Sample size	How sample size was determined
Inclusion criteria	Whether criteria for selecting herds/animals were defined
Random allocation	Whether a random allocation of experimental units was used
Characteristics of experimental units	Whether the herds/animals characteristics were defined
Previous Se status	Whether Se status was defined before the experiment
Intervention protocol	Whether the intervention was adequately defined
Protocol feasible to adopt	Whether the protocol was feasible to adopt under non-experimental conditions
Control group	Whether a control group was included in the trial
Hierarchical structure	Whether a hierarchical data structure was accounted for in the analysis
Statistical analysis	Whether the analysis was appropriate for the experimental design
Design of the study	
Experimental design	Randomized clinical trial, block design, randomized block design, incomplete block design, Latin square, observational study, other
Study population	Experimental unit, commercial unit, both
Production system	Housed-, pasture-based, other
Parity	Primiparous, multiparous cows, both
Type of production	Beef cattle, dairy cattle, dual purpose
Treated groups	Number of treated groups
Duration of the study	Months
Number of visits	Number of visits to collect milk samples
Selenium analysis	Graphite furnace atomic absorption spectroscopy, hydride generation atomic absorption spectroscopy, inductively coupled plasma/atomic emission spectroscopy, chromatography, neutron activation analysis, other
Source	Sodium selenite/selenate, selenium yeast
Dose	Dose of Se in mg/day
Stage	Stage of lactation when the study started
Frequency	Frequency of Se administration (once, continued)
Days	Number of days after treatment to sample collection
Interaction	Whether an interaction between treatment and time was significant

2.3.7   Influential Studies

Studies influencing the summary estimate were identified generating an influence plot. An influence graph was generated as a standard error bar chart, in which summary estimates were computed after omitting sequentially one study at a turn (Deeks et al., 2001), those studies having an undue influence on the estimation of the average effect of treatment were identified.

All analyses were carried out in Stata Statistical Software release 10.0 using the commands 'metan', 'metabias', 'metafunnel', 'metareg', 'metacum', and 'metaninf' (Stata Corp., College Station, TX, USA). No adjustment for clustering within author was made, due to a low number of studies performed by the same author.

2.4   Results

2.4.1   Literature Search

The search identified 139 potential references containing the keyword combination either in their titles or abstracts. A total of 23 references could not be recovered (1 narrative review, 4 duplicates of other studies, 3 written in English, and 15 written using a language beyond the scope of selected languages). From the remaining 116 references, 77 were excluded from the analysis (Appendix 1). In 3 manuscripts, the outcome of interest was described, but no data were recorded because supranutritional doses of Se were used; these reports were written in English, and their results are shown in Table 2.2.

Three manuscripts reported the results of 5 studies where milk Se concentration was evaluated at time points other than between 28 and 170 days from treatment and were therefore excluded from the meta-analysis (Table 2.3).

Table 2.2. Summary of 3 studies reported in 3 references not included in the meta-analysis.

Reference	Country[1]	n[2]	Parity[3]	Stage[4]	Source[5]	Dose (mg/cow/d)	Days[6]	Milk Se (μmol/L)	Mean diff.[7]	Sig.[8]	Reason[9]
Heard et al., 2004	AU	15	Mult.	E	Yeast	25.00	9	1.77	1.65	S	Unusual dose
Pehrson and Johnsson, 1985[i]	SE	18	Mult.	E	Na-Sel.	30.00	NR	NR	NR	NR	Unusual dose
Stagsted et al., 2005	DK	3	NR	NR	Yeast	25.00	30	1.77	1.60	NR	Unusual dose

[1]Country codes according to official short names in English as given in ISO 3166-1 and ISO 3166-1-alpha-2 code elements.
[2]Number of cows.
[3]Parity: Mult. = multiparous; All = first calving and multiparous; NR = not reported.
[4]Stage of lactation when the study started: D = dry period; E = from calving to 100 DIM; L = more than 100 DIM.
[5]Source of selenium: Na-Sel. = Sodium selenite/selenite; Yeast = Selenium yeast.
[6]Days: lag time from supplementation to first milk sample collection for Se analysis.
[7]Mean diff.: difference between means for milk Se concentration of supplemented and unsupplemented cows.
[8]Significance: NS = not significant, S = $P < 0.05$.
[9]Selenium was administered (30 mg/cow) two times during late pregnancy.

Table 2.3. Summary of 5 studies reported in 3 references not having analysis of milk selenium between 30 and 170 days from supplementation.

Reference	Country[1]	n[2]	Parity[3]	Stage[4]	Source[5]	Dose (mg/day)	Days[6]	Milk Se (μmol/L)	Mean diff.[7]	Sig.[8]
Fisher et al., 1980	US	3	NR	L	Na-Sel.	6.00	13	0.34	0.00	NS
Fisher et al., 1980	US	3	NR	L	Na-Sel.	12.00	13	0.42	0.06	NS
Jenkins et al., 1974	CA	8	Mult.	D	Na-Sel.	0.80	182	0.27	0.13	NS
Jenkins et al., 1974	CA	8	Mult.	D	Na-Sel.	0.80	210	0.22	0.17	NS
Sustala et al., 2003	CZ	4	Mult.	L	Yeast	10.20	21	0.56	0.37	S

[1]Country codes according to official short names in English as given in ISO 3166-1 and ISO 3166-1-alpha-2 code elements.
[2]Number of cows.
[3]Parity: Mult. = multiparous; All = first calving and multiparous; NR = not reported.
[4]Stage of lactation when the study started: D = dry period; E = from calving to 100 DIM; L = more than 100 DIM.
[5]Source of selenium: Na-Sel. = Sodium selenite/selenite; Yeast = Selenium yeast.
[6]Days: lag time from supplementation to first milk sample collection for Se analysis.
[7]Mean diff.: difference between means for milk Se concentration of supplemented and unsupplemented cows.
[8]Significance: NS = not significant, S = $P < 0.05$.

Consequently, 33 manuscripts containing the results of 42 studies provided data that fulfilled all criteria and were used to perform the meta-analyses. Twenty-eight manuscripts were published in peer-reviewed journals, three were published as abstracts, one appeared as part of conference proceedings, and one was published as a book chapter. Thirty-one reports were written in English, one in Portuguese, and one in German. These references were categorized according to the continent where they were performed: 15 were carried out in America (Canada, the United States, and Brazil), 13 in Europe, and 5 in Oceania (Australia and New Zealand).

Seven studies in 5 manuscripts did not report the SD (Ammerman, et al., 1980; Aspila, 1991; Syrjala Qvist and Aspila, 1993; Malbe, et al., 1995; Hemken et al., 1998); thereupon, their SD were imputed from all the other available studies, and included in the meta-analysis (Furukawa, et al., 2006). Out of the 33 references, 25 reported a positive effect of Se supplementation, 4 did not show a significant effect, and 4 did not report the significance of the effect. All these 4 manuscripts reported, however, a numerically positive effect of Se supplementation on milk Se concentration (Table 2.4).

2.4.2   Meta-Analysis

The average treatment effect obtained using the method-of-moments estimation was 0.16 μmol/L (95% CI: 0.12, 0.21), with a significant heterogeneity among studies. The variation in the difference of milk Se concentration attributable to the heterogeneity (I^2) was estimated at 99.7%, corresponding to a very strong between-study variation.

The average effect did not change when a meta-analysis was performed after removing those studies that did not report the SD (Table 2.5). Additionally, adjusting the overall variance by the VIF to summarize the milk Se concentration measured at different time points did not produce any change in either the average effect or on its 95% confidence interval (CI) (Table 2.5). The results of each trial are shown in Figure 2.1.

Table 2.4. Summary of the 42 studies reported in 33 references used in the meta-analysis.

Reference	Country[1]	n[2]	Parity[3]	Stage[4]	Source[5]	Dose (mg/cow/d)	Days[6]	Milk Se (μmol/L)	Mean diff.[7]	Sig.[8]
Ammerman et al., 1980	US	3	Mult.	D	Na-Sel.	1.26	133	0.16	0.04	NS
Aspila, 1991	FI	11	All	L	Na-Sel.	6.20	77	0.15	0.06	S
Batchelor, 2002	AU	8	Mult.	L	Yeast	2.40	45	0.82	0.60	S
Bis-Wencel, 2003	PL	20	Mult.	E	Na-Sel.	0.60	79	1.34	0.30	S
Brzoska and Brzoska, 2004	PL	8	Mult.	E	Na-Sel.	4.83	84	0.23	0.10	S
Charmley et al., 1993	CA	12	All	E	Yeast	5.00	56	0.44	0.27	S
Conrad and Moxon, 1979	US	5	NR	L	Na-Sel.	3.03	67	0.19	0.08	NR
Cuesta et al., 1993	US	21	Mult.	D	Na-Sel.	1.85	100	0.23	0.05	S
Gierus et al., 2002	DE	10	NR	E	Na-Sel.	3.47	49	0.17	0.03	S
Gierus et al., 2003	DE	20	Mult.	D	Na-Sel.	2.40	74	0.11	0.02	NS
Grace et al., 1997	NZ	20	Mult.	D	Na-Sel.	6.00	112	0.10	0.05	S
Guyot et al., 2007	BE	6	Mult.	D	Na-Sel.	5.84	85	0.38	0.13	S
Guyot et al., 2007	BE	6	Mult.	D	Yeast	5.84	85	1.65	1.39	S
Heard et al., 2007	AU	6	Mult.	L	Yeast	11.64	32	1.45	1.11	S
Heard et al., 2007	AU	6	Mult.	E	Yeast	10.94	32	1.26	1.10	S
Hemken et al., 1998	US	4	NR	E	Na-Sel.	3.10	70	0.65	-0.08	S
Hemken et al., 1998	US	4	NR	E	Na-Sel.	6.30	70	0.76	0.04	S
Hidiroglou and Proulx, 1988	CA	6	prim	D	Na-Sel.	2.70	120	0.11	0.04	S
Hidiroglou et al., 1985	CA	23	Mult.	D	Na-Sel.	3.00	114	0.16	0.06	S
Hidiroglou et al., 1987a	CA	49	All	D	Na-Sel.	4.00	90	0.16	0.01	NS
Hidiroglou et al., 1987b	CA	10	Mult.	D	Na-Sel.	2.50	120	0.12	0.06	S
Juniper et al., 2006	GB	20	Mult.	E	Na-Sel.	2.25	35	0.26	0.02	S
Juniper et al., 2006	GB	20	Mult.	E	Yeast	4.28	35	0.51	0.27	S
Knowles et al., 1999	NZ	7	NR	L	Na-Sel.	3.00	88	0.17	0.09	S
Knowles et al., 1999	NZ	7	NR	L	Yeast	3.00	88	0.51	0.43	S
Malbe et al., 1995	EE	4	NR	L	Na-Sel.	4.20	56	0.30	0.25	NR
Malbe et al., 1995	EE	4	NR	L	Yeast	4.20	56	0.81	0.75	NR

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McDowell et al., 2002	US	8	Mult.	D	Yeast	2.10	140	0.44	0.06	S
McIntosh and Royle, 2002	AU	3	Mult.	L	Yeast	4.00	42	0.26	0.17	S
Muniz-Naveiro et al., 2005	ES	12	NR	L	Na-Sel.	2.87	35	0.30	0.02	S
Muniz-Naveiro et al., 2005	ES	12	NR	L	Yeast	2.84	35	0.39	0.11	S
Ortman and Pehrson, 1999	SE	10	All	E	Na-Sel.	3.00	63	0.21	0.04	S
Ortman and Pehrson, 1999	SE	11	All	E	Yeast	3.00	63	0.40	0.23	S
Paschoal et al., 2007	BR	8	All	L	Yeast	5.00	56	0.40	0.05	S
Perry et al., 1977	US	3	Mult.	D	Na-Sel.	2.67	144	0.14	0.04	NR
Phipps et al., 2007	UK	10	Mult.	L	Yeast	4.95	112	0.84	0.53	S
Phipps et al., 2007	UK	10	Mult.	L	Na-Sel.	3.22	112	0.50	0.18	S
Salih et al., 1987	US	12	All	D	Na-Sel.	3.50	90	0.08	0.02	S
Stowe et al., 1988	US	38	Mult.	D	Na-Sel.	2.00	67	0.26	-0.02	NS
Syrjala Qvist and Aspila, 1993	FI	11	All	L	Na-Sel.	4.07	109	0.17	0.07	NR
Waldron et al., 2004	US	10	Mult.	L	Na-Sel.	7.50	42	0.11	0.03	S
Wiewiora et al., 2003	PL	8	Mult.	E	Na-Sel.	2.10	83	0.07	0.02	S

[1]Country codes according to official short names in English as given in ISO 3166-1 and ISO 3166-1-alpha-2 code elements.
[2]Number of cows.
[3]Parity: Mult. = multiparous; All = first calving and multiparous; NR = not reported.
[4]Stage of lactation when the study started: D = dry period; E = from calving to 100 DIM; L = more than 100 DIM.
[5]Source of selenium: Na-Sel. = Sodium selenite/selenite; Yeast = Selenium yeast.
[6]Days: lag time from supplementation to first milk sample collection for Se analysis.
[7]Mean diff.: difference between means for milk Se concentration of supplemented and unsupplemented cows.
[8]Significance: NS = not significant, S = $P < 0.05$.

Table 2.5. Average effect of oral selenium supplementation on milk Se concentration obtained after removing the studies which did not report the SD or after adjusting the overall variance by the variance inflation factor (VIF).

	n^1	T^2	95% CI[3]	$\tau^{2,4}$
Actual estimate	42	0.162	0.117, 0.207	0.0192
SD reported	35	0.163	0.114, 0.211	0.0192
Adjusting by VIF:				
$\rho = 0.90$[5]	42	0.161	0.116, 0.206	0.0185
$\rho = 0.75$	42	0.161	0.116, 0.206	0.0186
$\rho = 0.25$	42	0.162	0.117, 0.207	0.0190

[1]Number of studies.
[2]Average effect.
[3]95% confidence interval.
[4]Between-study variance.
[5]Intraclass correlation coefficient.

Study	Author
1	Ammerman et al., 1980
2	Aspila, 1991
3	Batchelor, 2002
4	Bis-Wencel, 2003
5	Brzoska and Brzoska, 2004
6	Charmley et al., 1993
7	Conrad and Moxon, 1979
8	Cuesta et al., 1993
9	Gierus et al., 2002
10	Gierus et al., 2003
11	Grace et al., 1997
12	Guyot et al., 2007
13	Guyot et al., 2007
14	Heard et al., 2007
15	Heard et al., 2007
16	Hemken et al., 1998
17	Hemken et al., 1998
18	Hidiroglou and Proulx, 1988
19	Hidiroglou et al., 1985
20	Hidiroglou et al., 1987a
21	Hidiroglou et al., 1987b
22	Juniper et al., 2006
23	Juniper et al., 2006
24	Knowles et al., 1999
25	Knowles et al., 1999
26	Malbe et al., 1995
27	Malbe et al., 1995
28	McDowell et al., 2002
29	McIntosh and Royle, 2002
30	Muniz-Naveiro et al., 2005
31	Muniz-Naveiro et al., 2005
32	Ortman and Pehrson, 1999
33	Ortman and Pehrson, 1999
34	Paschoal et al., 2007
35	Perry et al., 1977
36	Phipps et al., 2007
37	Phipps et al., 2007
38	Salih et al., 1987
39	Stowe et al., 1988
40	Syrjala-Qvist and Aspila, 1993
41	Waldron et al., 2004
42	Wiewiora et al., 2003
Overall	

Milk Se difference (μmol/L)

Figure 2.1. Forest plot of the effect of oral selenium supplementation on milk selenium concentration (μmol/L) difference in cattle. The average estimate of the effect was derived from the random-effects meta-analysis. The length of the horizontal line represents the 95% confidence interval (CI) for the effect size from each study, the center of the square (O) represents the point estimate from that study, and the area of the square is proportional to the weight assigned to the study. The dashed line is the average effect of treatment (0.16 μmol/L) obtained from the analysis, while the solid vertical line marks the value where Se supplementation would have no effect. The diamond (◇) at the bottom of the dashed line shows the 95% CI for the overall effect (0.12, 0.21), and the horizontal line beside the diamond represents the interval (95%: -0.17, 0.50 μmol/L) of the milk Se difference for future studies.

2.4.3 Publication Bias

The statistical approaches used for the evaluation of publication bias showed differing results. Begg's test reported a significant bias ($P < 0.001$), while the Egger's test did not suggest a significant bias ($P = 0.28$). The asymmetrical appearance (i.e. a gap in the lower left quadrant) in Figure 2.2 suggested that publication bias might be present. However, the average estimate, using the random-effects "Trim and Fill" method, did not result in any change in the effect of treatment obtained in the random-effects meta-analysis, and no missing studies were imputed.

2.4.4 Meta-Regression Analyses

None of the variables related to study quality (Table 2.1) recorded in the database showed a significant association with the outcome of interest. However, the effect of Se supplementation on milk Se concentration was less when cows were part of a randomized clinical trial ($P = 0.15$). The unconditional analyses showed no significant association (data not shown) of the outcome variable with those variables related to the study design (Table 2.1), such as study population, production system, type of production, parity, frequency of Se administration, or days from treatment to first sample collection. A significant ($P < 0.01$) unconditional association with the outcome of interest was found for the continent where the study was carried out and the source of Se (Table 6). The relationship of milk Se concentration to dose of Se was non linear, which was indicated by the significance of a quadratic term for dose (Table 2.6).
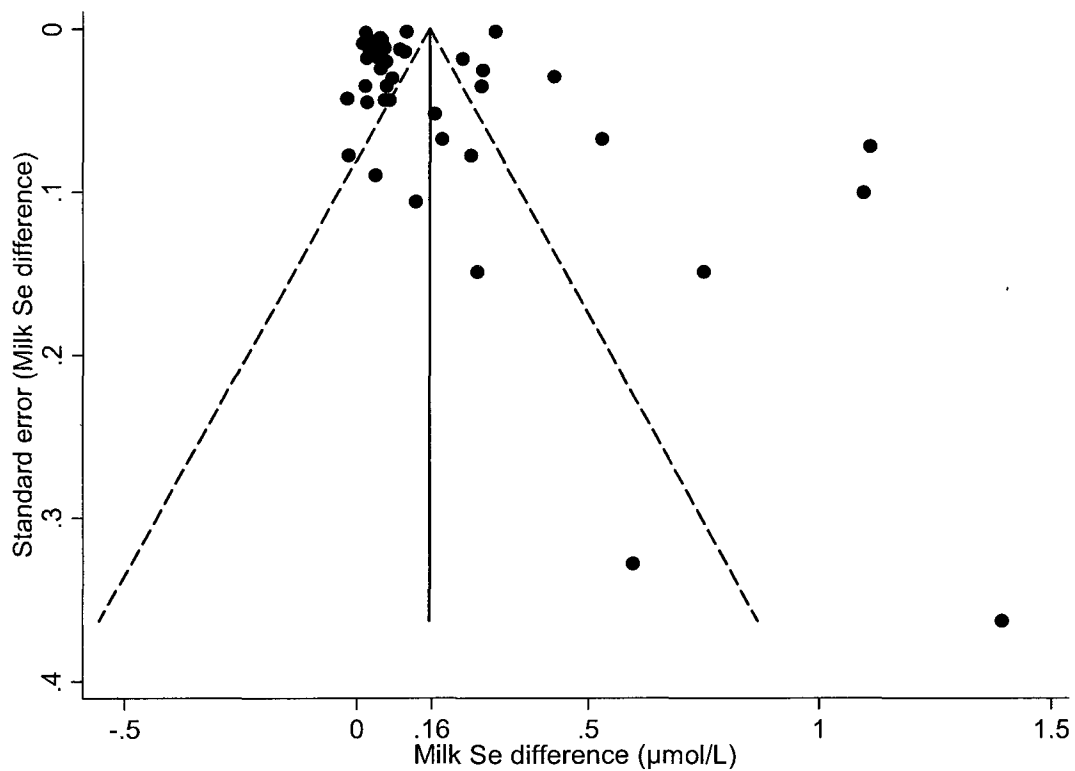
Figure 2.2. Funnel plot of the point estimates of the effect of oral selenium supplementation on the difference of milk selenium concentration (µmol/L) in cattle.

Significant predictors remained in the multivariable model (Table 2.7). Two interactions were not presented in the table. The interaction between continent and source of Se was close to significant ($P = 0.06$), and the interaction between source of Se and the quadratic effect of dose was not significant ($P = 0.48$). Those interactions were omitted in the model. There were two somewhat extreme residuals corresponding to two trials where Se yeast was used (Malbe et al., 1995; Guyot et al., 2007); their removal had little impact on estimates and consequently, those studies were retained in the model.

Milk Se concentration was predicted using the coefficients of the multivariable regression model, and plotted against several doses of Se in the form of sodium selenite/selenate and Se yeast (Figure 2.3). For example, studies that administered Se yeast (6 mg/cow/day) and carried out in America had, on average, a milk Se concentration of 0.37 μmol/L higher than the milk Se concentration in cows supplemented with sodium selenite/selenate 75 days after the beginning of supplementation.

A sensitivity analysis omitting two Australian trials that found high milk Se concentrations after supplementing with Se yeast was carried out. In those trials, Se yeast was given at a dose higher than 10 mg/cow/d (Heard et al., 2007). The analysis resulted in a non-significant effect of dose of Se, but curves were similar to those shown in Figure 2.3.

A decrease in milk Se concentration was evident at a rate of Se supplementation lower than 3 mg/cow/d; however, the effect depended upon Se source and continent. On average, sodium selenite/selenate was supplemented at a dose of 3.2 ± 1.6 mg/cow/d and Se yeast at 4.3 ± 1.4 mg/cow/d in American studies, while studies from Australia/New Zealand supplemented 4.5 ± 2.1 mg/cow/d and 6.4 ± 4.5 mg/cow/d of sodium selenate/selenite and Se yeast, respectively.

Table 2.6. Univariable meta-regression based on 42 studies of Se supplementation in cattle.

Factor	Coef.[1]	95% CI[2]	P-value	$\tau^{2,3}$
Null model	0.162	0.083, 0.241	< 0.01	0.019
Random allocation	-0.115	-0.276, 0.045	0.15	0.019
Continent			< 0.01	0.019
America[4]	0			
Europe	0.100	-0.051, 0.253	0.19	
Oceania[5]	0.379	0.167, 0.591	< 0.01	
Stage of lactation			0.13	0.011
Dry period[6]	0			
Early lactation (< 100 DIM)	0.133	-0.047, 0.314	0.14	
Late lactation (> 100 DIM)	0.168	-0.004, 0.339	0.06	
Milk yield (n = 9)[7]	-0.035	-0.094, 0.025	0.213	0.038
Source				0.027
Sodium selenite/selenate	0			
Selenium yeast	0.333	0.190, 0.476	< 0.01	
Dose	-0.102	-0.179, -0.026	0.01	0.005
Quadratic term for dose	0.015	0.008, 0.021	< 0.01	
Days	-0.002	-0.005, 0.000	0.08	0.019

[1]Coefficient.
[2]95% confidence interval.
[3]Method-of-moments estimator of the between-study variance (τ^2).
[4]Canada, the United States, and Brazil.
[5]Australia and New Zealand.
[6]Dry period corresponds to beginning Se supplementation before calving.
[7]Milk production was reported in only 9 studies.

Table 2.7. Multivariable meta-regression based on 42 studies of Se supplementation in cattle.

Factor	Coef.[1]	95% CI[2]	P-value	$\tau^{2,3}$
Null model	0.162	0.083, 0.241	< 0.01	0.019
Multivariable model			< 0.01	0.006
Intercept	0.001	-0.189, 0.191	0.99	
Continent			< 0.01	
America[4]	0			
Europe	0.118	0.043, 0.193	< 0.01	
Oceania[5]	0.116	0.010, 0.222	0.03	
Se yeast	-0.080	-0.297, 0.138	0.47	
Dose	-0.063	-0.124, -0.001	0.05	
Dose_sq	0.007	-0.000, 0.014	0.05	
Days	0.001	0.000, 0.003	0.04	
Interactions				
Source*dose	0.075	0.024, 0.127	< 0.01	

[1]Coefficient.
[2]95% confidence interval.
[3]Method-of-moments estimator of the between-study variance.
[4]Canada, the United States, and Brazil.
[5]Australia and New Zealand.
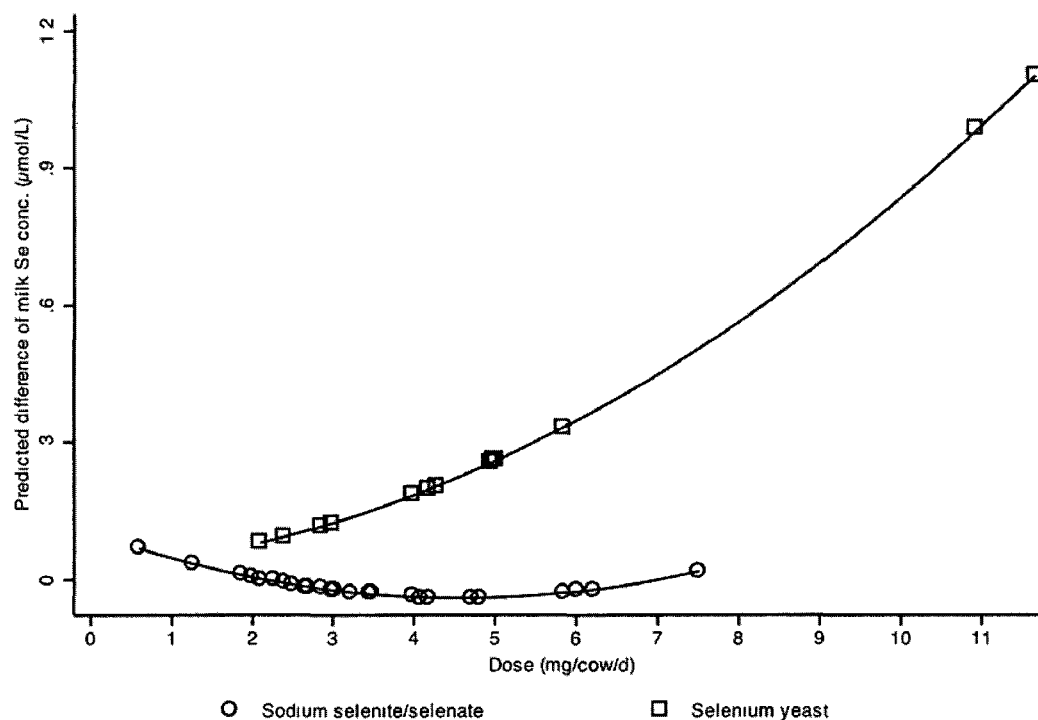
Figure 2.3. Effect of the dose of Se on milk Se concentration (μmol/L) 75 d after the beginning of supplementation, predicted according to the multivariable regression model for a study carried out in some American countries (e.g. Canada, the United States or Brazil). The symbols indicate the actual doses among the 42 studies.

2.4.5 Cumulative Meta-Analysis

The random-effects cumulative meta-analysis of the studies published until 2007 is presented in Figure 2.4. A repeated pooled estimate and its 95% CI after a sequential combination of the studies is displayed in ascending publication date order. The first point of interest is the significant effect of Se supplementation on milk Se concentration from the first trial published in 1977. The studies showed the lowest response to Se supplementation from 1977 (Perry et al., 1977) to early 90s (Syrjala Qvist and Aspila, 1993). At that time, 336 cows had been allocated to 11 studies for comparing the effect of sodium selenite/selenate supplementation (3.2 ± 1.3 mg/cow/d) against unsupplemented cows.

The average effect started to change gradually toward a higher milk Se difference after the inclusion of Se yeast as Se source in early 90s (Charmley et al., 1993). The results of the subsequent 31 studies which enrolled 631 additional cows increased the average effect of treatment, and its 95% CI became wider. Moreover, the dose of Se was related to publication year and Se source. After 1993, sodium selenite/selenate was used, on average, at a dose of 3.6 ± 1.8 mg/cow/d compared to 5.0 ± 2.8 mg/cow/d when Se yeast was supplemented.

2.4.6 Influential Studies

A simple sensitivity assessment was performed by repeating the meta-analysis but excluding individual studies one at a time (Figure 2.5). No individual studies had an undue influence on the pooled estimate. However, omitting study 4 (Bis Wencel, 2003) had the largest effect on the pooled estimate, and in this case the average treatment effect dropped from 0.16 μmol/L (95% CI: 0.12, 0.21) to 0.13 μmol/L (95% CI: 0.10, 0.15).
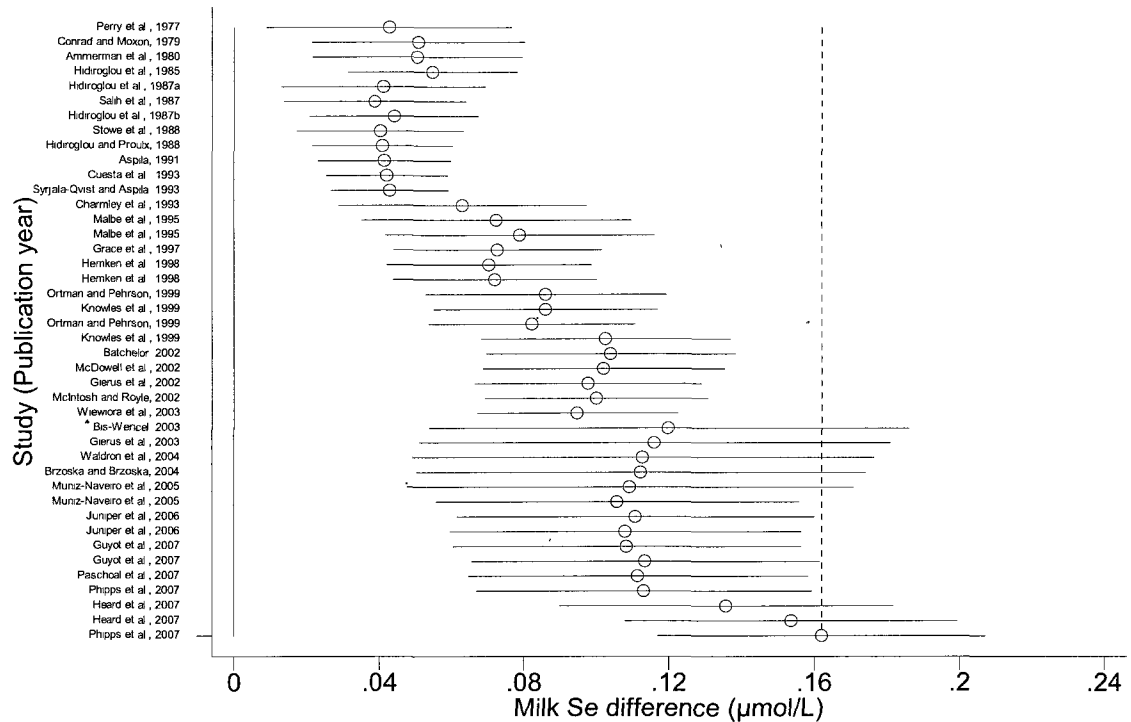
Figure 2.4. Cumulative meta-analysis of 42 studies of the effect of selenium supplementation on milk Se concentration (μmol/L) difference in cattle - ordering by year of publication. The figure displays repeated average effect estimates (O), and their 95% confidence intervals (horizontal line) for the analysis performed after adding each study. The solid vertical line marks the value where supplementation would have no effect, and the dashed vertical line marks the current average effect of treatment.
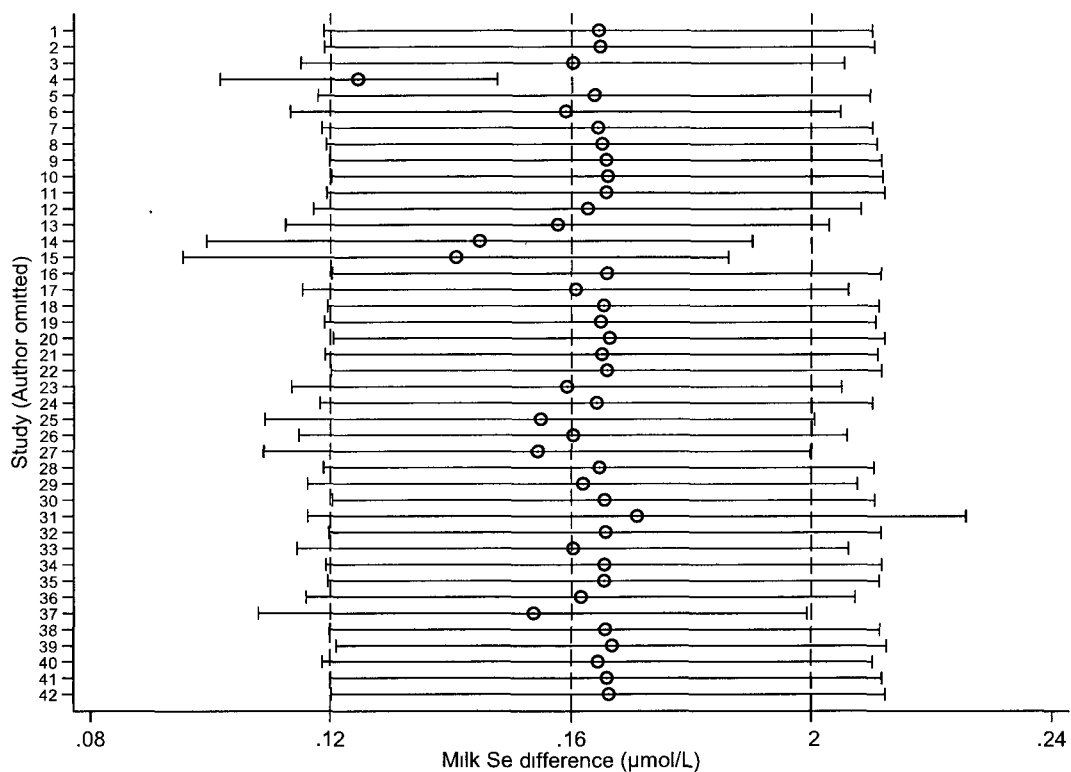
Figure 2.5. Influence plot of 42 studies of the effect of selenium supplementation on milk Se concentration (μmol/L) difference in cattle. The figure displays the average estimate (O) of the effect, and its 95% confidence interval (each horizontal line) for the meta-analysis repeated systematically excluding each individual study at a time (Refer to Figure 2.1 for study identification).

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2.5 Discussion

A meta-analysis based on the results of the 42 studies meeting all selected criteria estimated an average increase in milk Se concentration of 0.16 μmol/L in response to oral Se supplementation. However, results varied considerably among studies: continent, source of Se and dose of Se were significant contributors to this variation. Nonetheless, other factors such as study design, production system, previous Se status, type of production, supplementation protocol, duration of supplementation, and Se analysis methodology did not have a significant effect on the treatment response.

Two factors that might have contributed to the variation in supplementation response were stage of lactation and milk yield. Milk yield did not reduce the between-study variance. Stage of lactation was not significantly associated with the outcome, but explained 43% of the between-study variance. Milk yield was not reported in all studies and its effect was evaluated using the results of 11 studies. A recent study found that milk Se concentration was associated with stage of lactation, whereby early lactation cows had lower milk Se concentration than did late lactation cows. This effect was likely mediated by a simple dilution of milk yield (Wichtel et al., 2004), or perhaps by digestion kinetics, as higher DMI might decrease Se absorption.

The critical examination for the presence of publication bias, or other bias types, is an integral part of the meta-analysis process (Egger et al., 1997). In this case, visual assessment indicated weak evidence of the presence of publication bias, but Egger's test was not significant and the "Trim and Fill" test did not impute any study. The asymmetry observed in the funnel plot might be an indication of the inclusion of studies of lower quality (e.g. poor methodological design of small studies). On average, smaller studies are conducted and analyzed with less methodological rigor than are larger studies (Egger, et al., 1997). The effect of small studies on the assessment of publication bias has not gone unnoticed, and a significant exaggeration of the

treatment response has been observed when results of lower-quality trials were pooled (Moher et al., 1998). In this meta-analysis, the random allocation of the experimental units was weakly associated with the study outcome. The effect of Se supplementation on milk Se concentration tended to be smaller when cows were randomly allocated to the experimental groups (β = -0.12, P = 0.15), supporting the contention that smaller studies tend to overestimate the effect of the treatment (Egger, et al., 1997; Moher, et al., 1998). However, the average effect was not modified whether or not the study was published in peer-reviewed journals, which might reflect a better quality of the study.

With regard to the meta-regression analysis, in the univariate analysis supplementation with Se yeast resulted in a higher milk Se concentration compared to supplementing with sodium selenite/selenate. Studies where Se yeast was used tended to supplement at higher doses than did studies where inorganic sources were administered, and studies performed in Australia/New Zealand used even higher doses of both sources than did American or European studies. This is an indication that Se source might be affected by continent (i.e. confounding effect), since these variables were also related to milk Se concentration.

A lack of effect on predicted milk Se concentration observed when less than 3 mg Se/cow/d were given might be related to individual responses that caused a high variability in milk Se concentration across studies. Weiss (2005) described a linear relationship of Se dose to milk Se concentration, but no substantial changes were observed when the intake of inorganic Se increases. Based on this linear regression, a change in Se intake from 2 to 5 mg/cow/d when inorganic sources are supplemented would result in an increase in milk Se concentration of 0.06 μmol/L, but this association was not controlled for potential confounders. A low positive increase in milk Se concentration was also observed in a trial performed in England where incremental doses of inorganic Se were given (Givens, et al., 2004). In contrast, the meta-regression in this study showed that an increase from 2 to 5 mg/cow/d in Se intake when

inorganic Se is fed would cause a decrease in milk Se concentration of 0.04 μmol/L, and the magnitude and direction of that change is related to continent and the dose of Se as shown.

Particular characteristics of soils, forages, and cattle production in America and Europe compared to Australia/New Zealand, and Se yeast characteristics, may account for the different strategies used in the design of trials on Se supplementation. Hence, the suggestion that continent, source, and dose were associated and acted as potential confounders. Australia and New Zealand, where cattle production is pasture-based, have Se-deficient soils because of low pH and rainfall (Australia) or volcanic parent material (New Zealand), and both countries are acknowledged as pioneers in Se research in livestock (Oldfield, 2002). Factors such as the recognition of deficiency (Oldfield, 2002), previous data on low milk Se concentration from Australia (Heard, et al., 2004), England (Givens, et al., 2004), Estonia (Pehrson, et al., 1997), New Zealand (Grace, et al., 1997; Knowles, et al., 1999), Nordic countries (Ekholm, et al., 1991), and North America (Maus, et al., 1980), and small contribution of livestock-derived food products to the Se intake of humans from several countries (Combs, 2001) might be related to the choice of different doses for Se supplementation trials where the objective is to enhance the Se status in animals, and the intake of Se for consumers. Thus, Australian reports have described the use of supranutritional doses of Se yeast, trying to increase the content of Se in milk (Heard, et al., 2004; Heard, et al., 2007), while American studies have been carried out adjusting them to lower Se intakes reflecting NRC (2001) recommendations. Moreover, Se intake is legally restricted in North America but not in Australia/New Zealand, and this may also affect the choice for higher doses.

The biological properties of Se yeast may also account for the observed response in milk Se concentration. Recently, a narrative review suggested that Se concentration was increased by 90% when cattle were fed Se yeast compared to an inorganic source (Weiss, 2005). Selenium yeast is a dried nonviable yeast (*Saccharomyces cerevisiae*) product. The yeast is cultivated

using a fed-batch fermentation process that provides incremental amounts of Se salts, minimizing the detrimental effects of Se salts on the yeast. This allows for the optimal incorporation of inorganic Se into cellular organic material, acquiring similar properties to natural Se sources (Weiss, 2005). Organic Se yeast contains a variety of Se proteins, mainly selenomethionine (Se-met), and other low molecular weight seleno-compounds. Even though there is little information concerning Se yeast metabolism in ruminants, it appears that Se coming from Se yeast is probably better absorbed than Se inorganic forms (Weiss, 2005). *In vitro* studies found a better diffusibility of Se-met, which contributes to its high absorption *in vivo* (Shen et al., 1997). Moreover, Se yeast is better transferred to milk than inorganic Se sources (Knowles, et al., 1999; Pehrson, 2005; Juniper, et al., 2006), probably because of the amino acid composition of milk proteins. Milk has approximately two times higher methionine concentration when compared to blood protein. Therefore, it is two times more likely that Se-met will be incorporated into milk protein than blood protein (Weiss, 2005).

The recommended dietary intake of Se for humans depends upon gender and age but, on average, a daily intake between 26 and 55 μg meets the requirement for adults. This intake must be higher in pregnant or lactating women (Institute of Medicine, 2000; WHO/FAO, 2004). According to the meta-regression analysis, to provide at least 10% of the minimum recommended dietary intake for Se when 100 mL of milk will be consumed daily, cows in America should be fed at least 11 mg/cow/d of sodium selenite/selenate or 6 mg/cow/d of Se yeast to comply with the suggestion of the Institute of Medicine (2000), WHO/FAO (2004) and Canada Food Guide for daily Se intake.

The pattern observed after the cumulative meta-analysis, which was the change toward a greater effect, might have been the result of a combination of several factors, such as an improvement in study designs (i.e., use of more powerful statistical analysis); amendment to allowable Se supplementation in the United States in 1987, which allowed 0.3 mg/kg (on a DM

basis) of supplemental Se to be added to ruminant diets (Ullrey, 1992), the marketing of Se yeast which began in the early 90s, and approval for its use in cattle in 2003 (FDA, 2003). However, the effect of source across years might be confounded by other factors (e.g. previous Se status, age of the cow, stage of lactation, etc) that did not show any significant association with milk Se concentration in this study.

The average effect slightly decreased after the removal of study 4 (Bis Wencel, 2003). The effect dropped 18% but remained positive. That particular study was performed in Europe (Poland) using sodium selenate as Se source (0.6 mg/cow/d). A large increase of milk Se concentration was found in supplemented cows (0.30 μmol/L) compared to unsupplemented cattle. That study had a relatively large sample size (n = 20 cows), and the precision of the estimate was high (SE: 0.001 μmol/L), which is an indication of a strong influence on the average effect of treatment. However, this study was neither blinded nor were cows randomly allocated to treatments, and the cows in unsupplemented group had a higher milk Se concentration than cows from other trials. In spite of having a high milk Se concentration, the cows in this trial responded favourably to a low Se supplementation with inorganic selenium.

2.6 Conclusions

On average, an increase of 0.16 μmol/L in milk Se concentration might be expected after oral Se supplementation in cattle, and an effect on milk Se concentration between -0.17 and 0.50 μmol/L (95% certainty) might be expected in a future clinical trials conducted to evaluate the effect of oral Se supplementation on milk Se concentration. High variation between studies was observed due in part to geographic factors, and some characteristics related to the study design (e.g. stage of lactation, Se source, dose of Se, etc). There was weak evidence of

publication bias. The effect of the dose of Se was unexpectedly low when Se was given to cows at a dose less than 3 mg/cow/d. However, other studies reporting a linear relationship did not account for potential confounders (i.e. source of Se). Higher doses of Se (i.e. organic forms) are required to achieve an adequate milk Se concentration for human consumption according to the country where the study will be performed. The seleno-amino acids from Se yeast are metabolized by mechanisms distinct from those of inorganic forms, and may be the form of choice for enhancing milk Se concentration. The challenge is to tailor the Se form and supplementation protocols to meet animal dietary requirements and to benefit consumers of dairy products.

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3   MILK SELENIUM CONCENTRATION AND ITS ASSOCIATION WITH UDDER HEALTH IN ATLANTIC CANADIAN DAIRY HERDS[2]

3.1 Abstract

Soils and plants in Atlantic Canadian provinces are known to contain low concentrations of selenium (Se). Earlier studies have indicated that dairy producers in Atlantic Canada are providing insufficient supplementary Se in the ration to meet the Se requirements of dairy cattle, as measured by herd-level milk Se concentration. The objective of this study was to evaluate the association between milk Se concentration and somatic cell count (SCC), and the risk of new intramammary infection (IMI) in the dry period, in Atlantic Canadian dairy cows. Eighteen dairy farms participating in the Canadian Bovine Mastitis Research Network cohort study were selected as a convenience sample. On each farm 15 cows to be dried off were selected. Quarter milk samples were collected at four wk and two wk before drying-off, within 24 h after calving and at seven d after calving to evaluate IMI status. Composite milk samples were analyzed for SCC and Se concentration. Mean milk Se concentration was marginal in 14% of the cows that were on pasture in the grazing season. Milk Se concentration was not associated with the overall odds of new IMI in the dry period. Somatic cell count increased with milk Se concentration, even after adjusting for IMI status. The dairy population in this study had higher ranges for milk Se concentration, while ranges for prevalence of IMI, and SCC were lower, than those in studies where a negative relationship between Se status and udder health was first noted. Therefore, under the current management conditions of selected dairy herds in Atlantic Canada, milk Se concentration does not appear to be a principal determinant of udder health.

3.2   Introduction

Much research and effort has been dedicated to mastitis and its control strategies in dairy herds. Nevertheless, mastitis remains a major challenge to the worldwide dairy industry because it restricts farm profitability both directly and indirectly. Economic losses are due to decreased milk yield, costs of drugs, early culling, extra labor, discarded milk, and increased rate of cow replacement (Halasa et al., 2007). A nationwide study conducted in 106 Canadian dairy herds reported an overall mean and median incidence rate of clinical mastitis (CM) of 23 and 17 cases per 100 cow-years, respectively, with *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus uberis* and coagulase-negative staphylococci (CNS) being the most frequently isolated pathogens (Olde Riekerink et al., 2008). In Prince Edward Island (PEI), at least 74% of dairy herds have at least one cow with a *Staph. aureus* IMI (Olde Riekerink et al., 2006).

Suboptimal nutrition is a recognized management risk factor for infectious disease in cattle (e.g. mastitis); in particular, Se and vitamin E have been extensively studied in the context of their role in udder health (Smith et al., 1997). North American and European studies in dairy herds with good control of CM caused by major pathogens found a negative association between Se status and bulk tank SCC (BTSCC) (Erskine et al., 1987; Weiss et al., 1990; Ndiweni et al., 1991). A Canadian study found, however, that BTSe was not associated with herd-level udder health parameters (Wichtel et al., 2004).

Soils in many regions of the world, including Atlantic Canada, have low Se content; consequently, cattle fed forages and crops grown on soils containing less than 0.6 mg Se/kg may receive suboptimal dietary Se (Gupta and Winter, 1975; Winter and Gupta, 1979; Smart et al., 1981). Locally grown feeds in Atlantic Provinces do not provide enough Se to meet the requirements of cattle. A low Se concentration (i.e. 0.04 mg/kg DM) has been found in forages grown in PEI (Vokaty, 1991), which is lower than the current recommendation in diets for dairy

cows (NRC, 2001). Canadian surveys have indicated that Se deficiency is a nationwide problem (Cathcart et al., 1980; Fenimore et al., 1983; Campbell et al., 1995), finding the lowest Se status in cattle raised in eastern Canada (Hoffman et al., 1973). Recent studies evaluating Se status by BTSe have found that lactating cows in 59% of PEI dairy herds were either marginally or truly Se-deficient, as many producers are providing insufficient supplementary Se for lactating dairy cows (Wichtel et al., 2004).

Little is known about the status of Se and its association with udder health in Canadian dairy herds. In the study by Wichtel et al. (2004), milk Se was measured during lactation, but not at dry-off or at calving, two critical periods for udder health when supplementation may be inconsistent because of the transition in dietary management or reduced feed intake. Because no studies have been conducted in Atlantic Canada concerning the association of Se status as measured by individual cow milk Se concentration with mammary gland health, and because evidence indicates that the daily intake of Se in lactating dairy cows from Atlantic Canada region tends to be lower than the current NRC recommendation, we hypothesized that an adequate Se status (i.e. high cow-level milk Se concentration) may be associated with an improvement of measures of udder health (e.g. lower risk of having a new IMI in the dry period, and lower SCC around calving) in dairy cows from selected dairy herds in Atlantic Canada. The objective of this study was to evaluate the association of individual milk Se concentration with SCC and the risk of having a new IMI in the dry period in Atlantic Canadian dairy cows.

3.3   Material and Methods

This study was approved by the Animal Care Committee of the University of Prince Edward Island in accordance with the requirements of the Canadian Council on Animal Care.

3.3.1 Herd and Animal Selection

In total, 18 dairy farms from New Brunswick (NB; n = 5), Nova Scotia (NS; n = 5) and

PEI (n = 8) participating in the Canadian Bovine Mastitis Research Network (CBMRN) cohort

study were selected as a stratified convenience sample (Reyher et al., 2010). The selection

process was based on strata of low, intermediate, and high 12-mo rolling average BTSCC in

2006, reflecting BTSCC averages of < 150,000, from 150,000 to 300,000, and > 300,000

cells/mL, respectively. All herds had > 80% Holstein-Friesian cows, milked twice daily, and

were DHI members. The relative proportion of tie-stall vs. free-stall herds in the study was

reflective of the target population for each province. Dairy production systems were classified as

follows: herds with cows housed in tie-stall or free-stall barns over the year with no access to

pasture, and herds with cows on pasture in the grazing season (i.e. spring and summer). The

study was conducted from February 2007 to January 2008 (Reyher et al., 2010).

Cows were selected to participate in the study if they were to be dried off over the

following two months from the time of the first visit, and if the cows were expected to remain in

the herd until at least two wk after calving. On average, 14 cows (between 8 and 19 cows per

herd) met the selection criteria. The length of the dry period was, on average, 63 d (95% CI: 60,

66). Cows calved between April and December 2007.

3.3.2 Sampling and Data Collection

Farm personnel aseptically collected quarter milk samples of selected cows between

four and two wk before drying-off, and a second set of samples was collected from two wk

before drying-off to drying-off. Quarter milk samples from cows were collected from calving to

24 hours after calving, and were re-sampled seven d after the first sample after calving.

Additionally, the farmer was asked to aseptically collect milk samples from those quarters that

had physical signs of CM (any visual abnormality of udder or milk, with or without systemic

signs of disease) at the time the abnormality was noted. Immediately after collection, samples were frozen on farm, and sent to the Atlantic Veterinary College (Charlottetown, PE, Canada).

Two composite milk samples were created for SCC and milk Se analyses. For the pre-dry sample, an aliquot of each quarter milk sample collected two wk before drying-off was mixed, while an aliquot of each quarter sample collected seven d after calving was mixed to get the post-fresh sample. During the monthly visits of CBMRN technicians to the farms, a bulk tank milk sample for Se analysis was collected in 17 of 18 study dairy herds in April, July, October 2007 and January 2008, to represent spring, summer, fall, and winter periods.

A questionnaire with closed and semi-closed questions was administered at each farm at the end of the study period to gather information on Se supplementation practices. Specific herd data, such as BMSCC and herd size, and specific cow data, such as parity, milk yield and calving date were obtained from the regional DHI organization.

3.3.3   Laboratory Analysis

Bacteriological analyses of milk samples were performed according to protocols established by the CBMRN (Reyher et al., 2010). An aliquot of 0.01 mL was cultured from all quarter milk samples, counting colonies when $\leq$ 10/plate (equivalent to $\leq$ 1000 cfu/mL). Pathogen identification was as follows: *Staph. aureus* was identified by Gram stain, and double-zone hemolysis on blood agar. Coagulase-negative staphylococci were identified by Gram stain, and no hemolysis on blood agar; however, if colonies were $\geq$ 10/plate, coagulase and deoxyribonuclease degradation tests were used for identification. *Staphylococcus aureus* was confirmed by a positive reaction to deoxyribonuclease degradation test, while CNS were confirmed when negative to both tests. Streptococci were identified by Gram stain, and negative catalase test. Christie, Atkins, Munch-Petersen, esculin, hippurate, inulin, and raffinose tests were used for identification. When necessary, the API 20 Strep System (bioMérieux SA, Marcy

l'Etoile, France) was used to identify *Strep. uberis*. *Streptococcus dysgalactiae* was confirmed by a positive latex agglutination test. Gram-positive, and catalase positive rods were considered to be *Corynebacterium* spp. or *Bacillus* spp. *Escherichia coli*, and *Klebsiella* spp. were identified by Gram staining, being oxidase negative, and their typical appearance on MacConkey agar. Identification of genera was made after using urea, citrate, spot indole, and triple-sugar iron test. Other Gram-negative rods or cocci, and positive oxidase test were considered to be other Gram-negative bacteria.

Composite milk samples were refrigerated in preparation for SCC and Se analyses. Milk SCC was determined using a Foss 4000 cell counter (Foss Electric, Hillerød, Denmark). Milk Se concentration was evaluated by the graphite furnace atomic absorption spectroscopy method (Oster and Prellwitz, 1982). Readings were carried out using a spectrometer PerkinElmer AAnalyst 800 (PerkinElmer, Waltham, MS).

The milk Se concentration was expressed as micromoles/liter (μmol/L). Reference ranges were as follows: < 0.12 μmol/L is considered to represent inadequacy, while 0.20 μmol/L divided the marginal range into low- and high-marginal categories, and $\geq$ 0.28 μmol/L was taken to represent adequacy (Wichtel et al., 2004). Wichtel et al (2004) established the reference range for BTSe by a regression model of the mean herd serum Se concentration on the BTSe concentration of 15 PEI dairy herds with widely differing BTSe concentrations. The cut-off points for BTSe were selected by calculating the point where previous reference values for serum Se intersected the regression line of the model.

3.3.4   Definition of Intramammary Infection

A sample was considered culture-positive when $\geq$ 1 cfu/0.01 mL (equivalent to $\geq$ 100 cfu/mL) of any pathogen was isolated. A quarter was considered to have an IMI at drying-off when both samples collected at drying-off were culture-positive for the pathogen in question. A

quarter with an IMI at drying-off that was negative for both samples collected after calving was considered as cured over the dry period. An IMI was established in the dry period when a quarter sample was culture-positive for a pathogen in at least one of the samples collected after calving. An IMI established in the dry period was considered as new if both quarter samples collected at drying-off were culture-negative for the pathogen in question.

The number of recovered (i.e. cured) quarters was recorded. Samples containing more than two bacterial species were considered contaminated, and were not informative of IMI status. However, if *Staph. aureus* or *Streptococcus agalactiae* were found in a contaminated plate, the sample was considered culture-positive for any of those pathogens.

3.3.5 Statistical analysis

Separate analyses were carried out for milk Se concentration, occurrence of new intramammary infections, and SCC. The following general steps were part of all analyses. Unconditional associations between independent and dependent variables were established in models incorporating the relevant hierarchical data structure (as detailed below). All independent variables unconditionally associated ($P \leq 0.10$) with the dependent variables were included in a multivariable model which was manually reduced by backwards selection of the significant variables ($P \leq 0.05$). All analyses included the following independent variables: province (PEI, NB, and NS), season (winter (December 21st to March 20th), spring, summer and fall), housing system (free-stall or tie-stall barn), whether or not cows were on pasture in the grazing season, and parity (1, 2 to 3, > 3). First-order interactions of significant variables were also assessed. Significant associations were assessed by pairwise comparisons with a Bonferroni adjustment for multiple testing.

Milk Selenium. The dependent variable, milk Se concentration obtained from each of the two composite samples per cow (prior to drying-off and post calving), was analyzed by linear

mixed models with the previously listed independent variables. Clustering within herds and cows was accounted for by including herd random effects and a compound symmetry correlation structure within cows (Dohoo et al., 2009). The model assumptions were evaluated by examining the standardized residuals, and significant associations were represented by model-based least squares means. Linear mixed model analysis was carried out using the MIXED Procedure of SAS version 9.2 (SAS Institute Inc, Cary, NC, USA).

Incidence Risk of Intramammary Infection. The dependent variable, occurrence of a new IMI with a specific pathogen (or overall) after calving in a quarter not infected at drying-off, was analyzed by logistic models with the previously listed independent variables plus the cow-level milk Se concentration at drying-off. Clustering within cows and herd was accounted for by the alternating logistic regression algorithm (Kleinbaum and Klein, 2002; Dohoo et al., 2009). Significant associations were represented by odds ratios with a population-averaged interpretation across all cows and herds. Alternating logistic regression analysis was carried out using the GENMOD procedure of SAS version 9.2 (SAS Institute Inc, Cary, NC, USA).

Somatic cell count. The dependent variable SCC obtained from each of the two composite samples per cow (prior to drying-off and post calving), was analyzed (on natural log scale) by linear mixed models similar to those previously described for milk Se concentration, except that this latter variable was included as an independent variable. The impact of the cow-level IMI status at drying-off, defined by parallel interpretation of the corresponding quarter-level IMI status, was evaluated by fitting additional models with this independent variable included. Cow-level IMI status should be considered as an intermediate (intervening) variable for the association between milk Se and SCC; the two analyses therefore gave the total and direct effects of milk Se, respectively (Dohoo et al., 2009). An optimal power transformation of SCC was obtained by Box-Cox analysis (Dohoo et al., 2009) because the model assumptions for the analysis on log scale were not fully met; however, a log transformation of SCC values was

used in the final model (Ali and Shook, 1980).

3.4 Results

Cows were housed over the year in one herd using tie-stall barn, while cows had access to pasture in the remaining herds (n = 6) that also used tie-stall barns for housing them. A free-stall barn was used in 11 herds, with cows confined over the year in six herds. On average, cows grazed six mo of the year, ranging from three to eight mo.

Two PEI herds left the study before the completion of the after calving follow-up period; thus, the information on quarter samples collected after calving from 23 cows was not available for analysis (Appendix 2). Additionally, 14 cows did not calve during the study period, and quarter samples from seven cows were not collected after calving. The herd average number of lactating cows over the study period was 81, ranging between 42 and 197, with an average parity of 3, with values ranging from 1 to 9. The average daily milk yield was 31.6 ± 3.5 kg/cow.

The 12-mo rolling average BTSCC ranged from 95,000 to 328,000 cells/mL, with a geometric mean of 173,000 cells/mL across all herds over the study period. Rolling average for two dairy herds was < 150,000, between 150,000 and 300,000 for 13 herds, and > 300,000 cells/mL for 3 herds.

3.4.1 Milk Selenium

All farmers reported the inclusion of Se in the ration for bred heifers, dry and lactating cows all year round. Adding Se to concentrates was the preferred method for supplementation. Three producers reported use of additional supplementation with a free-choice mineral block, a top dress for pasture, and an injectable preparation at drying-off (one herd each). Mean and

standard deviation of BTSe were 0.52 ± 0.12 µmol/L. Only two samples from two different herds had a BTSe < 0.28 µmol/L.

A total of 427 composite milk samples from 262 cows in 18 dairy herds were included in the final analysis. Mean individual milk Se concentration was 0.52 ± 0.17 µmol/L, with eight cows (3%) having a milk Se concentration < 0.28 µmol/L. No differences were observed in milk Se concentration due to province, parity or stage of lactation, while the unconditional analysis indicated a seasonal variation, and differences due to the housing system, and whether or not cows were allowed to graze (Table 3.1). Neither housing system nor the interactions between season and grazing, and housing system and grazing were associated with milk Se concentration, and were removed from the final model (Table 3.2). There was a significant seasonal variation of milk Se concentration, and highest milk Se concentration was found in cows confined all year round (Table 3.2).

The correlation between two different cows within the same herd was calculated, a fairly strong value was found ($\rho = 0.43$).

3.4.2 Intramammary Infection

The association of milk Se concentration with the odds of new IMI in the dry period were evaluated using 3816 quarter milk samples from 262 cows in 18 Atlantic Canadian herds. At drying-off, 2080 quarter milk samples were collected and cultured; of these, 17.4% were contaminated (Table 3.3), and omitted from further analysis. Only 1736 quarter milk samples were collected after calving because of the cows lost to follow up; the proportion of contaminated samples was 12% (Table 3.3).

Table 3.1. Unconditional associations obtained from a linear mixed model for milk selenium concentration (μmol/L) in 427 composite samples from 262 cows in 18 Atlantic Canadian dairy herds.

Variable	LSM[1]	95% CI[2]	P-value
Province			0.24
Prince Edward Island	0.49	0.40, 0.58	
New Brunswick	0.49	0.37, 0.60	
Nova Scotia	0.60	0.49, 0.71	
Season			< 0.01
Fall	0.53	0.46, 0.61	
Winter	0.58	0.49, 0.66	
Spring	0.55	0.49, 0.62	
Summer	0.47	0.41, 0.53	
Housing system			0.02
Free-stall barn	0.57	0.50, 0.64	
Tie-stall barn	0.44	0.36, 0.53	
Grazing			< 0.01
No	0.58	0.52, 0.64	
Yes	0.47	0.41, 0.53	
Parity			0.16
1	0.53	0.46, 0.59	
2 - 3	0.53	0.47, 0.59	
> 3	0.50	0.44, 0.57	
Stage of lactation			0.59
Fresh	0.52	0.45, 0.58	
Drying-off	0.53	0.46, 0.59	

[1]Least square means.
[2]95% confidence interval.

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Table 3.2. Multivariable linear mixed model estimates of the associations of season and grazing with milk selenium concentration (μmol/L) in 427 composite samples from 262 cows in 18 Atlantic Canadian dairy herds.

Variable	Mean[1]	95% CI[2]	*P*-value
Season			< 0.001
Fall	0.51[a]	0.44, 0.59	
Winter	0.60[a,b]	0.52, 0.69	
Spring	0.65[b]	0.58, 0.73	
Summer	0.58[a]	0.50, 0.66	
Grazing			< 0.001
No	0.62[a]	0.54, 0.69	
Yes	0.45[b]	0.38, 0.51	

[1]Adjusted (least square) mean not including grazing for season effect, and only including spring and summer seasons for grazing effect.
[2]95% confidence interval.
[a,b]Different superscripts differ significantly (adjusted *P* < 0.05).

Table 3.3. Quarter sample distribution of mastitis pathogens at drying-off and after calving in 3816 quarters from 262 cows in 18 dairy herds from Atlantic Canada.

Sample status	Drying-off		After calving	
	No.	%	No.	%
Culture-negative	887	42.6	919	52.9
Culture-positive	832	40.0	611	35.2
Staphylococcus aureus	50	2.4	59	3.4
Staphylococcus hyicus	1	0.0	3	0.2
CNS	490	23.6	299	17.2
Streptococcus spp.[1]	71	3.4	50	2.9
Streptococcus dysgalactiae	3	0.1	7	0.4
Streptococcus uberis	14	0.7	6	0.3
Escherichia coli	2	0.1	7	0.4
Enterobacter spp.			13	0.7
Corynebacterium spp.	47	2.3	20	1.2
Klebsiella spp.			3	0.2
Nocardia spp.	1	0.0	6	0.3
Other Gram-positive pathogens[1]	145	7.0	124	7.1
Other Gram-negative pathogens[2]	1	0.0	7	0.4
Yeast	5	0.2	2	0.1
Fungi	2	0.1	5	0.3
Contaminated	361	17.4	206	11.9
Total	2,080		1,736	

[1] *Strep. agalactiae, Strep. uberis, Strep. dysgalactiae,* and *Strep. canis* were excluded from this category.
[2] *Bacillus* spp.
[3] *Serratia* spp., *Citrobacter* spp., *Proteus* spp., *Salmonella* spp., *Pseudomonas* spp., and *Pasteurella multocida.*

At drying-off, 40% of the quarter milk samples were culture-positive. Coagulase-negative staphylococci were the most frequently isolated group of bacteria, followed by other Gram-positive bacteria, and *Streptococcus* spp. (Table 3.3). After calving, 35% of the quarter milk samples were culture-positive, with CNS, other Gram-positive bacteria, *Staph. aureus*, and *Streptococcus* spp. being the most common isolations (Table 3.3).

The quarter-level prevalence of IMI at drying-off was 22% (i.e. both pre-dry samples positive), with CNS IMI being the most frequently isolated bacteria (70%), followed by *Staph. aureus* (12%), other Gram-positive pathogens (9%), and *Streptococcus* spp. (4%). The proportion of IMI that cured over the dry period was 39%, ranging from 21 to 100% depending upon the pathogen (Table 3.4). The incidence of new IMI in the dry period was 59%; of these CNS IMI were 41%, followed by other Gram-positive pathogens IMI (21%), *Streptococcus* spp. IMI (13%), and *Staph. aureus* IMI (9%) (Table 3.4).

The unconditional association of milk Se concentration with the odds of new *Staph. aureus* IMI and CNS IMI was not significant (Table 3.5). However, the odds of new *Streptococcus* spp. IMI unconditionally increased with increasing milk Se concentration. An increase of 0.20 μmol/L in milk Se concentration was associated with 1.88 times higher odds of having a new *Streptococcus* spp. IMI (Table 3.5). Adjusting the association of milk Se concentration with the odds of new pathogen-specific IMI for the effect of other variables produced only minor changes in the coefficients (Table 3.6).

3.4.3   Somatic Cell Count

The association of individual milk Se concentration with SCC was based on 313 composite milk samples, as contaminated samples were removed from the analysis. Geometric mean SCC was 56,000 cells/mL, with samples ranging from 3,000 to 8,240,000 cells/mL. The unconditional associations of LnSCC with all independent variables are presented in Table 3.7.

Table 3.4. Number of intramammary infections (IMI) at drying-off (n = 730 quarters), cured IMI over the dry period, and new IMI in the dry period (n = 440 quarters).[1]

Pathogen	IMI at drying-off		Cured[2]		New IMI in dry period	
	No.	%	No.	%	No.	%
Staphylococcus aureus	19	2.6	4	21	23	5.2
CNS	112	15.3	43	38	106	24.1
Streptococcus spp.[3]	6	0.8	3	50	33	7.5
Streptococcus dysgalactiae	1	0.1	1	100	4	0.9
Streptococcus uberis	4	0.5	1	25	4	0.9
Escherichia coli	-	-	-	-	5	1.1
Enterobacter spp.	-	-	-	-	2	0.5
Corynebacterium spp.	4	0.5	4	100	9	2.0
Klebsiella spp.	-	-	-	-	3	0.7
Nocardia spp.	-	-	-	-	4	0.9
Other Gram-positive pathogens[4]	14	1.9	6	43	55	12.5
Other Gram-negative pathogens[5]	-	-	-	-	4	0.9
Yeast	-	-	-	-	2	0.5
Fungi	1	0.1	1	100	5	1.1
Total	161	22.1	63	39	259	58.9

[1]Contaminated samples were omitted from the analysis
[2]Cured IMI over the dry period.
[3]*Strep. agalactiae*, *Strep. uberis*, *Strep. dysgalactiae*, and *Strep. canis* were excluded from this category.
[4]*Bacillus* spp.
[5]*Serratia* spp., *Citrobacter* spp., *Proteus* spp., *Salmonella* spp., *Pseudomonas* spp., and *Pasteurella multocida*.

Table 3.5. Unconditional effect of increasing milk selenium concentration in 0.2 μmol/L, province, housing system, grazing, and parity on the odds of having pathogen-specific new intramammary infection in the dry period in 440 quarters from 192 dairy cows from 16 Atlantic Canadian dairy herds.

Variable	*Staphylococcus aureus*			*Streptococcus* spp.[1]			CNS		
	OR[2]	95% CI[3]	P-value	OR[2]	95% CI[3]	P-value	OR[2]	95% CI[3]	P-value
Milk Se	0.61	0.00, 7.40	0.28	1.88	1.12, 3.14	0.02	0.98	0.73, 1.30	0.86
Province[4]			0.10			0.01			0.02
PEI	1			1			1		
NB	0.30	0.05, 1.67		2.12	0.87, 5.20		0.61	0.42, 0.87	
NS	1.50	0.28, 8.00		3.19	1.42, 7.19		0.82	0.41, 1.64	
Housing system			0.36			0.06			0.85
Free-*stall*	1			1			1		
Tie-stall	2.06	0.44, 9.57		0.55	0.30, 1.02		0.95	0.58, 1.57	
Grazing			0.79			0.01			0.39
No	1			1			1		
Yes	1.28	0.21, 7.66		0.45	0.25, 0.82		0.78	0.43, 1.39	
Parity			< 0.01			0.99			0.30
2 - 3	1			1			1		
> 3	2.92	1.64, 5.23		1.00	0.48, 2.06		1.26	0.81, 1.96	

[1]*Strep. agalactiae, Strep. uberis, Strep. dysgalactiae,* and *Strep. canis* were excluded from this category.
[2]Odds ratio.
[3]95% confidence interval.
[4]Prince Edward Island, New Brunswick, and Nova Scotia.

Table 3.6. Effect of an increase of 0.20 µmol/L in milk selenium concentration on the pathogen-specific and overall odds of having a new intramammary infection during the dry period in 440 quarters from dairy cows from Atlantic Canadian dairy herds.

Pathogen	OR[1]	95% CI[2]	P-value
Staphylococcus aureus[3]	0.49	0.18, 1.29	0.15
Streptococcus spp.[4]	1.72	1.03, 2.85	0.04
CNS[4]	0.98	0.72, 1.34	0.91
Other Gram-positive pathogens[5]	1.54	1.02, 2.32	0.04
Overall[3]	1.23	0.95, 1.59	0.11

[1]Odds ratio.
[2]95% confidence interval.
[3]Final multivariable model including: milk Se, province and parity.
[4]Final multivariable model including: milk Se and province.
ag and S canisNot including:
[5]Final multivariable model including: milk Se and housing system.

Table 3.7. Unconditional effect of milk selenium concentration, province, season, housing system, grazing, parity, stage of lactation, and infection status on the somatic cell count (LnSCC, thousands/mL) in 492 milk samples from 262 dairy cows in 18 Atlantic Canadian dairy herds.

Variable	Coef.[1]	95% CI[2]	P-value
Milk Se (μmol/L)	0.16	-0.00, 0.33	0.05
Province			0.90
Prince Edward Island	0		
New Brunswick	0.03	-0.46, 0.51	
Nova Scotia	-0.08	-0.56, 0.40	
Season			0.11
Fall	0		
Winter	0.71	-0.14, 1.56	
Spring	0.67	0.13, 1.22	
Summer	0.55	0.02, 1.08	
Housing system			0.96
Free-stall	0		
Tie-stall	-0.01	-0.41, 0.39	
Grazing			0.98
No	0		
Yes	0.00	-0.40, 0.40	
Parity			< 0.01
1	0		
2 - 3	0.48	0.11, 0.85	
> 3	-0.17	-0.52, 0.17	
Stage of lactation			< 0.01
Fresh	0		
Drying-off	0.82	0.57, 1.08	
Infection status			0.02
Negative	0		
Major pathogen	0.76	0.21, 1.31	
CNS	0.39	-0.08, 0.87	

[1]Coefficient.
[2]95% confidence interval.

The model validation showed a right-skewness in the residuals that could be eliminated using power transformation (optimal power of -0.25 obtained by Box-Cox analysis). However, the conclusions of the effect of milk Se concentration on SCC either after power transformation (not shown) or after log transformation were qualitatively similar. Therefore, results are presented using the log transformation for simplicity of the interpretation.

Two multivariable models, either including or not including the cow-level IMI status as an independent variable, were fitted (Table 3.8). Both models indicated a positive association of milk Se concentration with mean LnSCC; the analysis of power-transformed SCC confirmed this finding (not shown). However, the coefficient for milk Se concentration increased slightly (~ 10%) and was still positive after controlling for infection status (Table 3.8), indicating a direct association of milk Se with LnSCC.

The multivariable linear regression model indicated a lower mean LnSCC in the spring compared to the summer ($P < 0.01$). Primiparous cows had lower LnSCC than multiparous cows ($P < 0.01$), and there were no significant differences associated with parity ($P = 0.24$). Mean LnSCC was higher at drying-off than in fresh cows (Table 3.8). The IMI status also affected LnSCC, a lower LnSCC was found in negative IMI cows compared to cows having an IMI ($P < 0.01$). Cows with major pathogen IMI had higher LnSCC than cows with CNS IMI ($P < 0.01$). Although there was a significant effect of season, parity and stage of lactation on LnSCC, their interactions with milk Se concentration were not significant, with P-values of 0.84, 0.87, and 0.65, respectively.

Table 3.8. Multivariable linear regression model of the association between milk selenium concentration, season, parity, stage of lactation and infection status, and somatic cell count (LnSCC, thousands/mL) in milk of 313 milk samples from 262 dairy cows in 18 Atlantic Canadian dairy herds.[1]

Variable	Not including infection status			Including infection status		
	Coef.[1]	95% CI[2]	P-value	Coef.[1]	95% CI[2]	P-value
Intercept	1.91	1.21, 2.61	< 0.01	1.11	0.17, 2.06	0.02
Milk Se (μmol/L)	1.20	0.46, 1.94	< 0.01	1.33	0.42, 2.23	< 0.01
Season			0.02			0.01
Fall	0			0		
Winter	-0.06	-0.85, 0.72		0.24	-0.80, 1.28	
Spring	-0.13	-0.65, 0.40		0.02	-0.56, 0.59	
Summer	0.35	-0.15, 0.85		0.58	0.05, 1.11	
Parity			< 0.01			< 0.01
1	0			0		
2 - 3	0.58	0.22, 0.95		0.52	0.03, 1.01	
> 3	1.33	0.94, 1.73		1.33	0.81, 1.86	
Stage of lactation			< 0.01			< 0.01
Fresh	0			0		
Drying-off	1.38	1.06, 1.71		1.55	1.16, 1.94	
Infection status						< 0.01
Negative				0		
Major pathogen				0.86	0.37, 1.36	
CNS				0.65	0.22, 1.08	

[1]Coefficient.
[2]95% confidence interval.

3.5 Discussion

Bulk tank milk Se is an adequate indicator to monitor the Se status in lactating cows, with BTSe $\geq$ 0.28 μmol/L reflecting Se adequacy (Wichtel et al., 2004). Using this cut-off, two PEI herds where cows had access to pasture had marginal BTSe values in the summer, suggesting that Se intake was insufficient to meet the Se requirement in the grazing season.

Milk Se concentration in North American studies have ranged from 0.12 (Debski et al., 1987) to 0.77 μmol/L (Olson and Palmer, 1984). A Canadian study on individual milk Se concentration reported a mean of 0.35 μmol/L in cows fed Se-adequate diets (Fisher et al., 1980). In our study, 27% of the cows had milk Se levels < 0.35 μmol/L in the summer; of these, 64% were cows housed in tie-stall barns with access to pasture in the grazing season. Seasonal effects may be explained by a dilution effect. Even though this effect seems plausible, it is unlikely, because milk yield in the spring was 5% less than that in the fall, while the difference in milk Se concentration between those seasons was 28%. Consequently, seasonal changes in milk Se concentration are more likely associated with a sub-optimal intake due to low content, seasonal changes of Se content in Atlantic pastures, forages and grains (Gupta and Winter, 1975; Winter and Gupta, 1979), and differences in the amount of concentrate fed to dairy cows.

Approximately 90% of the cows had an adequate Se status as indicated by their milk Se concentration, most likely associated with the widespread recent adoption of improved Se supplementation practices in Atlantic herds. Although milk Se concentration changes rapidly in response to Se intake, not all methods for Se supplementation are equally efficacious (Wichtel, 1998; Wichtel et al., 2004; Chapter 2), with dose and source of Se closely related to the response in milk Se concentration. On average, American cows fed organic Se have 0.37 μmol/L more milk Se than cows supplemented with inorganic forms (Chapter 2). Milk Se concentration increases as organic Se intake increases, and organic Se is better transferred to milk than

inorganic sources (Chapter 2). Therefore, an enhanced milk Se concentration in Atlantic

Canadian herds as compared to former studies (Fisher et al., 1980; Wichtel et al., 2004) may be a

consequence of increased organic Se supplementation.

In all recent studies on prevalence of IMI and incidence of CM, CNS have been the most

frequently isolated pathogens, while the most frequently found major pathogen was *Staph.

aureus* (Piepers et al., 2007; Olde Riekerink et al., 2008; Sampimon et al., 2009). Coagulase-

negative staphylococci and *Staph. aureus* were the most common pathogens isolated in our

study, concurring with previous results. Other major pathogens (e.g. *Streptococcus* spp. and

other Gram-positive pathogens) were also frequently isolated.

The results of our study concur with a former study indicating no relationship of Se

status with the overall incidence of IMI and CM (Kommisrud et al., 2005). However, different

odds were found for the association of milk Se with pathogen-specific incidence of IMI. The

incidence of *Streptococcus* spp. IMI in the dry period increased with an increase of 0.2 μmol/L

in milk Se concentration. The odds of new *Staph. aureus* IMI or CNS IMI were not affected by

milk Se concentration, suggesting that the association of milk Se concentration at drying-off

with the odds of new IMI in the dry period might be pathogen-specific.

The effect of milk Se on the pathogen-specific odds may be due to pathogen-associated

differences in the polymorphonuclear (PMN) influx to the udder. Selenium-supplemented cows

had a massive and rapid PMN influx to the udder after *E. coli* IMI (Erskine et al., 1989). In case

of *Staph. aureus* IMI, a slower PMN influx was found in Se-suplemented cows, but the killing

ability of PMN was more efficient, suggesting some evidence of enhanced mammary resistance

to *Staph. aureus* IMI (Erskine et al., 1990). European studies have found that Se

supplementation resulted in a Se-dependent antibacterial activity in whey sufficient to inhibit the

growth of *Staph. aureus* (Ali-Vehmas et al., 1997; Malbe et al., 2006). Nevertheless, the odds of

having a *Streptococcus* spp. IMI at the beginning of lactation or a *Streptococcus* spp. CM during

lactation were not affected by Se supplementation (Smith et al., 1984; Smith et al., 1985). In our study, however, the reason for the effect of milk Se concentration on the odds of new IMI caused by *Streptococcus* spp. or other Gram-positive pathogens in the dry period remains unclear, but may, however, be related to specific PMN function.

Selenium effect on udder immune response is mediated through its incorporation into antioxidant selenoproteins (Sordillo et al., 2007); however, reaching a certain level of milk Se concentration does not guarantee an effective incorporation into selenoproteins with a role against mammary pathogens, as approximately 15% of the daily Se intake is excreted in milk (Maus et al., 1980). This observation concurs with a recent trial reporting an increased milk Se concentration after Se supplementation, but differences did not translate into an enhanced effect on PMN function or into the clinical response following an intramammary challenge with lipopolysaccharides (Weiss and Hogan, 2005).

Milk Se concentration was positively associated with mean LnSCC, concurring with previous studies from Norway (Kommisrud et al., 2005) and Canada (Wichtel et al., 2004), possibly as a result of a more robust PMN response to IMI in well Se-supplemented cows. A positive association of milk Se concentration with SCC was found in Se-supplemented cows following an intramammary challenge with lipopolysaccharides (Weiss and Hogan, 2005), as PMN migration following an IMI is improved in Se-adequate cows (Erskine et al., 1989). Additionally, bovine blood neutrophils have an extremely high activity of the selenoenzyme glutathione peroxidase, and blood cells contain a greater proportion (~ 73%) of blood Se than plasma (Scholz and Hutchinson, 1979). Therefore, an increase of SCC in milk may also cause an increase in milk Se concentration due to PMN influx into infected quarters.

Generally, SCC increases with advancing age and stage of lactation (Barkema et al., 1999; Olde Riekerink et al., 2007), with stage of lactation explaining more variation in SCC than does parity (Schepers et al., 1997). In our study, older cows had numerically more IMI caused by

Staph. aureus, streptococci and Gram-positive pathogens, which cause a higher increase in SCC compared to changes observed in CNS IMI or *Corynebacterium* spp. IMI (Schukken et al., 2003). The low SCC observed shortly after calving was, possibly, the consequence of a decline in the prevalence of infection (Dohoo, 1993) or a physiological effect in culture-negative quarters (Barkema et al., 1999).

In 2003, the approval of organic Se for use in dairy cattle greatly expanded the options for Se supplementation, making Se supplementation a more complicated matter, as differences in the bioavailability and the effect of Se sources on measures of herd productivity, and udder health became more variable. Therefore, producers who have typically higher SCC, might intentionally supplement Se at a higher rate, or may use more organic Se, resulting in a higher concentration in milk (Chapter 2) associated with higher SCC. Nevertheless, more supplemental Se does not mean a higher incorporation into the functional selenoproteins with a role in the immune response of the udder. Evidence coming from recent studies does not point to an obvious effect of supplementing organic sources compared to inorganic sources on udder health (Malbe et al., 1995; Weiss and Hogan, 2005). Selenium supplementation may elicit a favorable response, but super-supplementation of Se-adequate diets will not produce additional benefits to animal performance (Silvestre et al., 2007). Higher Se intakes may have evolved over time because producers and advisers have become more aware of the importance of maintaining adequate Se status. Thus, in the present study we were working with a dairy population where the ranges of milk Se concentration, the prevalence of IMI, and SCC in milk were much more favorable than in the experimental herds 25 years ago where a negative relationship between Se status and udder health was first established.

3.6   Conclusions

Seasonal variations in milk Se concentration indicated that mean milk Se was marginal in 14% of the cows in the grazing season, most likely the consequence of an insufficient Se intake due to low Se concentrations in pastures of Atlantic Canada. Although milk Se indicated an adequate Se status, overall odds of new IMI in the dry period was not associated with milk Se concentration. However, milk Se was positively associated with higher odds of new *Streptococcus* spp. IMI, and IMI caused by other Gram-positive pathogens in the dry period. Milk Se concentration was also positively associated with LnSCC, possibly as a result of a more robust PMN response to IMI in well supplemented cows. Selenium status was much higher, and ranges of IMI prevalence and SCC were lower in the population of our study than in the experimental herds where a beneficial role of Se status and udder health was first noted. Under the current management conditions, and ranges of milk Se levels found, Se status did not appear to be a principal determinant of incidence of IMI in the dry period and SCC changes shortly after calving; thus, management practices and environmental factors other than Se status determined the udder health.

3.7   Acknowledgements

technicians who collected the samples.

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3.8   References

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4 BULK TANK MILK SELENIUM AND ITS ASSOCIATION WITH MILK PRODUCTION PARAMETERS IN CANADIAN DAIRY HERDS[3]

[3] Ceballos, A. H. W. Barkema, H. Stryhn, I. R. Dohoo, G. P. Keefe, and J. J. Wichtel. 2010. Bulk tank milk selenium concentration and its association with milk quality in Canadian dairy herds.

4.1 Abstract

Low selenium (Se) concentrations have been found in soils and forages in Canada. Suboptimal Se intake has been associated with impaired performance and increased rates of disease in dairy cattle, especially with regard to udder health and milk quality. An observational study was conducted in 63 Canadian dairy farms that participated in the Canadian Bovine Mastitis Research Network, to evaluate the association of bulk tank milk Se concentration (BTSe) with measures of milk production parameters (i.e. daily milk yield, milk fat and milk protein), bulk tank somatic cell count (BTSCC), and the probability of being a *Staphylococcus aureus*-positive herd. Bulk tank milk samples, collected between March 2007 and February 2008, were evaluated for BTSCC, culture status with *Staph. aureus*, and BTSe. Mean BTSe was 0.51 ± 0.15 µmol/L; no herds were classified as deficient or marginal based on BTSe. Bulk tank milk Se was not associated with daily milk yield, milk fat and milk protein, or BTSCC. Higher values of BTSe were associated with lower risk of being a *Staph. aureus*-positive herd, possibly as a result of a more robust udder immune response to mammary pathogens, or as a result of providing Se at a higher rate as a management intervention to control BTSCC.

4.2 Introduction

Soils of the eastern and western Canadian coasts contain relatively low Se concentrations, while central and prairie provinces are largely Se-adequate for ruminant livestock production (Oldfield, 2002). Early surveys conducted in British Columbia (Fenimore et al., 1983) and Atlantic Canada (Winter and Gupta, 1979) have indicated a mean forage Se concentration of < 0.05 mg/kg DM, which has been described as the minimum concentration in

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forages for livestock production (Underwood and Suttle, 2001). Approximately 90% of forage samples from Atlantic Canada (Winter and Gupta, 1979), and 50% from Ontario were below that cut-off point (Young et al., 1977). A low Se concentration in forage has been associated with marginal serum Se concentrations in ruminants (Fenimore et al., 1983). Taken together, these results indicate that Se deficiency may be a widespread problem in Canada, particularly in the coastal provinces where livestock are fed entirely on locally grown feeds.

The evaluation of the production response to mineral supplementation has remained the most practical tool for assessing the micronutrient status of livestock (Wichtel, 1998). Milk yield has been claimed to be a sensitive indicator of Se status in dairy cattle, thus a low milk yield may be an important economic consequence of sub-clinical Se deficiency in cattle. In an early New Zealand trial, a 4.6% increase in milk yield was found in Se-supplemented cows (Fraser et al., 1987). In an study conducted in Prince Edward Island (PEI), Canada, dairy herds providing Se in concentrates were over four times more likely to be Se-adequate than herds not using this method, and daily milk yield was, on average, 7.6% higher in Se-adequate herds (Wichtel et al., 2004). Further, two North American clinical trials have indicated an increase between 2% and 5% in milk yield in dairy cows supplemented with organic Se at the onset of lactation (Silvestre et al., 2007).

Selenium has been extensively studied in the context of its role in udder health and milk quality. A dietary Se concentration < 0.1 mg Se/kg dry matter (DM) has been associated with an increased susceptibility to Se-responsive disorders in cattle, such as mastitis (Smith et al., 1997), and that risk can be even higher when the dietary Se concentration decreases to < 0.05 mg/kg DM (NRC, 1983; NRC, 2001). A number of North American herd-level studies have identified a negative association between Se status and bulk tank SCC (BTSCC) in dairy herds with good control of clinical mastitis (CM) caused by major pathogens (Erskine et al., 1987; Weiss et al., 1990). *Staphylococcus aureus* and *Streptococcus agalactiae* are the most frequently isolated

pathogens in herds with high BTSCC (Barkema et al., 1998). Herd-level prevalence of *Staph. aureus* was 83% in Canada, with a higher geometric mean BTSCC in *Staph. aureus*-positive than in negative herds (Olde Riekerink et al., 2006). However, the overall odds of having an IMI with any of those pathogens has not been associated with individual milk Se concentration (Chapter 3). A former study conducted in PEI also found no association of bulk tank milk Se concentration (BTSe) with any measure of udder health (Wichtel et al., 2004).

Wichtel et al. (2004) found that 59% of PEI dairy herds were at some point marginal for Se status. However, these results cannot be extrapolated to other Canadian provinces due to differences in the mineral content of soils and crops, and differences in mineral management practices. In addition, Se management practices have changed over the last decade, and more Se soruces are available, and little is known about the association between herd-level Se status measured by BTSe, and udder health in Canadian dairy herds. Because no studies of the association of BTSe with milk yield and udder health have been conducted in Canada, and because of some previous evidence indicating that daily Se intake in dairy cows may be lower than the current NRC (2001) recommendation, we hypothesized that Se status may be associated with milk production parameters and measures of udder health. The objectives of this study were: 1) to determine the Se status in selected Canadian dairy herds by measuring BTSe; and 2) to evaluate the association between BTSe concentration and milk production parameters, BTSCC, and the probability of being a *Staph. aureus*-positive herd.

4.3 Materials and Methods

A total of 63 dairy farms from Alberta (n = 15), Ontario/Québec (n = 31), and Atlantic Canada (n = 17) participating in the Canadian Bovine Mastitis Research Network (CBMRN)

cohort study were selected as a stratified convenience sample, and intended to represent as closely as possible the Canadian commercial dairy farm population. Selection criteria, production data, and housing management have been described elsewhere (Dufour et al., 2010; Reyher et al., 2010). Briefly, the selection process was based on the relative contribution of each region to Canadian milk production, and on strata of low, intermediate and high 12-month rolling average BTSCC in 2006, with cut-offs < 150,000, between 150,000 and 300,000, and > 300,000 cells/mL, respectively. The farms were grouped according to the dairy production system as follows: intensively managed herds with cows housed in tie-stall or free-stall barns over the year, and intensively managed herds with cows on pasture during the grazing season (i.e. between spring and summer). All farms had > 80% Holstein-Friesian cows, milked twice daily, and subscribed to DHI recording (Reyher et al., 2010).

4.3.1 Sampling and Data Collection

Study sampling procedures were discussed between the CBMRN personnel and dairy producers before the beginning of the study (Reyher et al., 2010). Technicians from the CBMRN project aseptically collected bulk tank milk samples from the selected farms on a monthly basis. Immediately after collection, samples were frozen and sent to the Maritime Quality Milk laboratory of the University of PEI (Charlottetown, PE, Canada) for analysis. Samples were cultured for *Staph. aureus*, and then preserved with bronopol and refrigerated or re-frozen in preparation for SCC analysis. An aliquot (5 mL) of the samples collected in April, July, October of 2007 and January 2008, which typically represented spring, summer, fall and winter periods, respectively, was used to evaluate BTSe concentration. However, a full set of the seasonal samples from each herd was not always available for BTSe analysis (Appendix 3).

Specific farm data, such as herd size (number of lactating cows), average DIM, and daily milk yield corresponding to the study period (i.e. from March 2007 to February 2008) were obtained from the regional DHI organizations.

4.3.2 Laboratory Analysis

Bacteriological analyses of bulk tank milk samples were performed following standardized protocols that were based on guidelines for bacteriological culture, and *Staph. aureus* identification (Hogan et al., 1999). For each bulk tank sample, an aliquot of 0.05 mL was plated on blood esculin agar to detect *Staph. aureus*. Plates were examined after 24 and 48 h of incubation, then colonies were counted when ≤ 10/plate (equivalent to ≤ 200 cfu/mL). *Staphylococcus aureus* was identified by Gram stain, a positive catalase test, and double-zone hemolysis on blood agar.

Preserved samples were analyzed for SCC using a Foss 4000 cell counter (Foss Electric, Hillerød, Denmark).

Milk Se concentration in fresh samples was evaluated by graphite furnace atomic absorption spectroscopy (Oster and Prellwitz, 1982). Readings were carried out using a spectrometer PerkinElmer AAnalyst 800 (PerkinElmer, Waltham, MS). The milk Se concentration was expressed as micromoles per liter (μmol/L). Herd Se status was interpreted using the cut-off points suggested by Wichtel et al. (2004). Briefly, the reference range for BTSe was established by fitting a regression model of the mean herd serum Se concentration on the BTSe concentration in 15 dairy herds from PEI with widely differing BTSe concentrations. The cut-off points for BTSe were selected by calculating the point where the reference values for serum Se from the peer-reviewed literature intersected the regression line of the model. Thus a BTSe concentration < 0.12 μmol/L was considered to represent deficiency, 0.20 μmol/L divided

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the marginal range into equally high- and low-marginal, and 0.28 μmol/L or higher was taken to represent an adequate status.

4.3.3 Statistical analysis

Bulk Tank Milk Selenium Concentration. A total of 192 samples were collected to evaluate BTSe; however, due to the uneven yearly distribution of samples within herds, a single annual BTSe value was estimated for each herd from the available data for the period between March 2007 and February 2008. The estimation was based on a multivariable linear mixed model for BTSe, including all independent variables: region, season (winter (Dec. 21st to Mar. 20th), spring, summer and fall), housing system (tie-stall, free-stall, and bedding pack barns), and whether or not cows had access to pasture in the grazing season. Clustering within herds was accounted for by including herd random effects (Dohoo et al., 2009).

Milk Production Parameters and Probability of Staphylococcus aureus-*Positive Herd.* The dependent variables used in the statistical models were: yearly average of daily milk yield, BTSCC (log scale), and isolation of *Staph. aureus* in bulk tank milk samples during the study period (March 2007 to February 2008). Milk production parameters and the probability of being a *Staph. aureus*-positive herd were analyzed by fixed-effects linear and logistic regression models, with region and the single estimated BTSe value as predictors. A *Staph. aureus*-positive herd was defined by the isolation of *Staph. aureus* (i.e. ≥ 1 cfu/0.05 mL) in two consecutive bulk tank milk samples collected no more than two months apart during the study period (slightly modified from Jayarao et al., 2004).

All model assumptions were evaluated by examining the standardized residuals. A natural logarithmic transformation of SCC values (1,000 cells/mL) was used to approximate the normal distribution (Ali and Shook, 1980). Analyses were carried out in SAS version 9.2 (SAS Institute Inc, Cary, NC, USA) using the MIXED, GLM, and LOGISTIC procedures.

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4.4 Results

4.4.1 Bulk Tank Milk Selenium Concentration

The majority of farms used a tie-stall barn system (57%), while free-stall and bedding-pack barn systems were used in 35% and 8% of the farms, respectively. Cows were housed throughout the year in 71% of the farms. Most of the farms (56%) where cows had access to pasture during the grazing season were located in Atlantic Canada. Some herd-level measures of productivity are presented in Table 4.1.

No BTSe values compatible with Se deficiency were found; however, eight samples (4%) were considered marginal (i.e. BTSe < 0.28 μmol/L). These marginal values were found in one farm from Québec, and in seven farms from Atlantic Canada; all of them used a tie-stall barn with cows having access to pasture in the grazing season. Five out of eight BTSe marginal values were found in the summer, two in the spring, and one in the winter.

Bulk tank milk Se concentration tended to differ among the three Canadian regions (Table 4.2). Bulk tank milk Se significantly differed between seasons, housing systems, and grazing. Mean BTSe was significantly lower in the summer, in farms using tie-stall barns, and in farms where lactating cows had access to pasture (Table 4.2).

The estimated yearly BTSe values were lower than the observed values (Table 4.3). Most of the bulk tank samples from dairy farms in Alberta and Ontario/Québec were collected in the winter and spring (Appendix 3), corresponding to the time of the year with higher BTSe values; therefore, the estimated yearly average values were lower than the observed averages. This difference was less marked in Atlantic Canada where herds had BTSe data collected in the summer and fall.

Table 4.1. Measures of herd productivity in 63 Canadian dairy farms between March of 2007 and February of 2008.

Variable	Mean	SD	Median	Range
Herd size				
Lactating cows	73	42	59	30 - 251
Dry cows	13	8	11	3 - 39
Culled cows	3	2	3	1 - 15
Average days in milk	200	16	196	176 - 268
Daily milk yield (kg/cow)	31.4	3.1	31.2	23.8 - 40.4
Milk fat (%)	3.7	0.2	3.7	2.4 - 4.2
Milk protein (%)	3.2	0.1	3.2	3.0 - 3.4
BTSCC (1,000 cells/mL)	219	77	211	81 - 416
Calving to first service (days)	75	19	72	26 - 166
Calving to conception (days)	129	29	130	35 - 221

[1]Bulk tank somatic cell count.

Table 4.2. Final multivariable regression model of the association between bulk tank milk selenium concentration and region, season, housing system, and whether or not cows had access to pasture in 63 dairy farms from three Canadian regions.

Effect	Coef.[1]	95% CI[2]	*P*-value
Intercept	0.53	0.38, 0.68	< 0.01
Region			0.10
Atlantic Canada	0		
Alberta	-0.01	-0.09, 0.06	
Ontario/Québec	-0.07	-0.14, -0.00	
Season			< 0.01
Fall	0		
Winter	0.11	-0.01, 0.23	
Spring	0.16	0.03, 0.28	
Summer	0.03	-0.10, 0.16	
Housing system	.		< 0.01
Bedding pack	0		
Tie-stall	-0.15	-0.26, -0.05	
Free-stall	-0.20	-0.30, -0.09	
Grazing			0.04
No	0		
Yes	-0.07	-0.14, 0.00	

[1]Coefficient.
[2]95% confidence interval.

Table 4.3. Average bulk tank milk selenium concentration (BTSe in μmol/L) estimated for 63 dairy farms from three Canadian regions.

Region	No. farms	BTSe observed		BTSe estimated	
		Mean	SD	Mean	SD
Alberta	15	0.53	0.17	0.45	0.09
Ontario/Québec	31	0.49	0.12	0.38	0.04
Atlantic Canada	17	0.52	0.18	0.47	0.08

4.4.2   Milk Production Parameters and Probability of Staphylococcus aureus-Positive Herd

An unconditional positive association between mean daily milk yield and BTSe was found (Table 4.4). Daily milk yield increased 12.3 kg for each unit (μmol/L) increase in BTSe ($P = 0.01$). However, no association was found between BTSe and other milk production parameters, such as milk fat and milk protein (Table 4.4). The association between BTSe and milk production parameters was adjusted by geographical region, leading to substantial changes in the coefficients (Table 4.4), and thus suggesting confounding effects of region on the relationship between BTSe and production.

Bulk milk Se concentration had no effect on BTSCC ($P = 0.29$), while regional differences were found (Table 4.5). Farms in Ontario/Québec had a higher mean BTSCC than did farms in Alberta ($P = 0.05$), and in Atlantic region ($P = 0.03$). No differences were observed between Alberta and Atlantic regions ($P = 0.91$). The adjustment of the association between BTSe and BTSCC by the effect of geographical region showed a substantial change in the coefficient, suggesting confounding effects of region on that relationship (Table 4.5).

Staphylococcus aureus was found at least once in 47 (75%) herds during the study period. Bulk tank milk Se concentration was unconditionally associated with the odds of being a *Staph. aureus*-positive herd ($P = 0.002$). For a BTSe increase of 0.20 μmol/L, the odds of being a *Staph. aureus*-positive herd changed by 0.04 units (Table 4.6). After controlling for region, the odds of being a *Staph. aureus*-positive herd increased, but a significant protective effect was still found. Higher values of BTSe were associated with lower risk of being a *Stap. aureus*-positive herd (Table 4.6).

Table 4.4. Unconditional associations and multivariable model of estimated bulk tank milk selenium concentration (BTSe in μmol/L) and region with yearly average of milk production parameters in 63 dairy farms from three Canadian regions.

	Daily milk yield (kg/d)			Milk fat (%)			Milk protein (%)		
	Coef.[1]	95% CI[2]	P-value	Coef.[1]	95% CI[2]	P-value	Coef.[1]	95% CI[2]	P-value
Unconditional associations									
BTSe	12.3	2.9, 21.8	0.01	-0.15	-0.9, 0.6	0.68	-0.16	-0.45, 0.12	0.26
Region			0.05			< 0.01			0.02
Alberta	0			0			0		
Ontario/Québec	-2.9	-4.8, -1.1		0.19	0.05, 0.32		0.05	-0.00, 0.11	
Atlantic Canada	-1.2	-3.2, 0.9		0.01	-0.14, 0.16		-0.02	-0.08, 0.04	
Multivariable model									
Intercept	30.0	25.0, 35.1	< 0.01	3.35	2.98, 3.72	< 0.01	3.16	3.01, 3.31	< 0.01
BTSe	7.0	-3.8, 17.8	0.20	0.62	-0.18, 1.14	0.13	0.08	-0.25, 0.40	0.64
Region			0.05			< 0.01			0.03
Alberta	0			0			0		
Ontario/Québec	1.1	-4.4, -0.5		0.23	0.08, 0.37		0.06	-0.00, 0.12	
Atlantic Canada	-1.3	-3.3, 0.7		0.00	-0.15, 0.15		-0.02	-0.08, 0.04	

[1]Coefficient.
[2]95% confidence interval.

Table 4.5. Unconditional association and multivariable model of estimated bulk tank milk selenium concentration (μmol/L) and region with log bulk tank milk somatic cell count (BTSCC) in 63 dairy farms from three Canadian regions.

	BTSCC (1,000 cells/mL)		
	Coef.[1]	95% CI[2]	P-value
Unconditional associations			
BTSe	-0.62	-1.78, 0.54	0.29
Region			0.05
Alberta	0		
Ontario/Québec	0.22	0.00, 0.44	
Atlantic Canada	-0.01	-0.26, 0.23	
Multivariable model			
Intercept	5.09	4.46, 5.72	< 0.01
BTSe	0.19	-1.14, 1.53	0.77
Region			0.08
Alberta	0		
Ontario/Québec	0.23	-0.01, 0.47	
Atlantic Canada	-0.02	-0.27, 0.23	

[1]Coefficient.
[2]95% confidence interval.

Table 4.6. Unconditional associations and final multivariable model for an increase of 0.20 μmol/L in bulk tank milk selenium concentration (BTSe) and region with the probability of being a *Staphylococcus aureus*-positive herd.

	OR[1]	95% CI[2]	*P*-value
Unconditional associations			
BTSe	0.035	0.004, 0.288	< 0.01
Region			< 0.01
Alberta	1		
Ontario/Québec	21.8	3.7, 127.2	
Atlantic Canada	2.8	0.7, 11.5	
Multivariable model			
BTSe	0.054	0.004, 0.704	0.03
Region			0.02
Alberta	1		
Ontario/Québec	11.2	1.7, 72.0	
Atlantic Canada	5.0	0.9, 28.4	

[1]Odds ratio.
[2]95% confidence interval.

4.5 Discussion

Bulk tank milk Se concentration responds rapidly to changes in Se intake, and has been satisfactorily used as a screening test for evaluating herd-level Se adequacy (Wichtel et al., 2004). Only eight samples out of 192 had BTSe < 0.28 µmol/L, seven out of these eight samples were from farms in Atlantic Canada. Those marginal values may be related to unusually low Se concentrations are found in forages and crops grown in the Atlantic Provinces (Winter et al., 1973), where approximately 90% of forage samples contain < 0.05 ppm of Se (Winter and Gupta, 1979), which is insufficient to meet the Se requirement in cattle. No BTSe values compatible with Se deficiency were found.

Researchers in the United States have reported mean milk Se concentrations in cattle ranging from 0.09 to 1.11 µmol/L (Olson and Palmer, 1984; Debski et al., 1987; Sanz and Diaz, 1995). In Canadian studies, mean cow-level milk Se concentration was 0.35 µmol/L (Fisher et al., 1980), and ranged between 0.23 and 0.34 µmol/L in herd-level studies (Wichtel et al., 2004). Seasonal variations have been found in Canadian studies, with greater risk of finding Se deficiency in the fall and winter compared to other seasons (Wichtel et al., 2004). Nevertheless, our results concur with those of researchers from the United States (Miller et al., 1995) and Europe (Norrman, 1984) who also found marginal Se values in the summer. A seasonal variation in Se concentration in pastures, and differences in the amount of concentrate fed to dairy cows may partially explain the seasonal changes found in BTSe.

The widespread adoption of improved Se supplementation practices by Canadian dairy managers, and the greater availability and use of organic forms of Se (e.g. Se yeast) in dairy rations, may, in part, explain an apparent reduction in the prevalence of low BTSe values in the current study when compared to earlier ones. Milk Se concentration changes rapidly in response to Se intake, but not all sources for supplementing Se are equally efficacious (Chapter 2). Milk

Se levels are higher when organic Se is fed compared to feeding inorganic sources (Chapter 2). Organic Se is better transferred to milk than inorganic sources (Knowles et al., 1999; Weiss, 2005; Juniper et al., 2006); therefore, a higher mean BTSe might be associated with the practice of using more organic Se for supplementation compared to alternative sources fed to cattle in earlier studies.

No association between BTSe and yearly average of daily milk yield was found in our study after adjusting for the confounding effect of region. Milk yield can be a sensitive indicator of Se status, and reduced milk yield may be the most economic consequence of marginal Se status (Wichtel, 1998). However, our results concur with former observational studies (Kommisrud et al., 2005; Enjalbert et al., 2006), and Se supplementation trials (Whelan et al., 1992; Coe et al., 1993; Grace et al., 2001) that found no association between Se status and milk yield parameters. Contrary to our results, milk yield and milk fat were higher in Se-adequate herds (i.e. blood Se > 0.15 μmol/L) compared to Se-deficient herds in New Zealand studies; additionally, milk yield in response to Se supplementation was higher when Se levels were low before supplementation (Fraser et al., 1987; Ellison, 1992). Mean BTSe in the herds of our study was higher compared to milk Se concentration in other studies where a positive response to Se supplementation was found, which may explain the lack of association between BTSe and milk yield.

There was no association between BTSe and BTSCC in our study, concurring with results found in Norwegian (Ropstad et al., 1987; Kommisrud et al., 2005), New Zealand (Grace et al., 1997), and North American (Lean et al., 1990; Wichtel et al., 2004) studies. Nevertheless, other studies have reported a negative association between Se status and BTSCC in herds with geometric mean BTSCC > 750,000 cells/mL (Erskine et al., 1987) or > 250,000 cells/mL (Weiss et al., 1990). The dairy population in our study had a Se status much higher than that in studies where a negative association between Se and udder health was first described. A high BTSCC

might induce the farmer to intentionally supplement Se at a higher rate resulting in a higher BTSe. This latter situation has resulted in observations of weak relationships between impaired udder health and Se status (Enjalbert et al., 2006). Moreover, geometric mean BTSCC in our dairy population was also much lower (196,000 cells/mL) than that in dairy herds 25 years ago, where a negative association between Se status and udder health was first described.

An increase of 0.20 μmol/L was negatively associated with the odds of being a *Staph. aureus*-positive herd in our study. Earlier studies found that an adequate Se status was associated with a decreased prevalence of mastitis pathogens, and lower BTSCC (Erskine et al., 1987; Weiss et al., 1990). Further, a protective effect of supplementing minerals, especially Se, on udder health and leukocyte function has been found (Piepers et al., 2009). Even though biomarkers of Se status (e.g. selenoproteins with specific functions) were not evaluated in our study, a reduction in the odds of being a *Staph. aureus*-positive herd can be explained by the biological function of Se on the immune system.

The effect of Se against mammary pathogens is mediated through several mechanisms: more rapid and massive influx of polymorphonuclear cells (PMN) to the udder (Erskine et al., 1989), bacteria (e.g. *Staph. aureus*) are killed more efficiently by PMN (Erskine et al., 1990), improved lymphocyte proliferation (Cao et al., 1992), high anti-bacterial activity in whey sufficient to inhibit *Staph. aureus* growth (Ali-Vehmas et al., 1997), and high expression of selenoproteins with antioxidant properties in the mammary gland (Sordillo et al., 2007; Sordillo and Aitken, 2009). These mechanisms, acting together in Se-adequate cows, enhance the immune response of the udder against mammary pathogens, reducing their herd prevalence.

Current mean BTSe was higher than that reported for Atlantic Canadian dairy herds earlier (Wichtel et al., 2004). The effectiveness of Se supplementation practices may have increased over time (Enjalbert et al., 2006). In addition, herds with a higher BTSCC (and higher odds of being *Staph. aureus*-positive) often supplement with Se, or provide Se at a higher rate,

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as a management intervention to control BTSCC.

The results of this study largely support the contention that, whereas supplementing cows consuming Se-deficient diets may elicit a favorable production response or a reduction in disease prevalence, but super-supplementation of Se-adequate diets may not result in additional benefits to animal performance (Silvestre et al., 2007). Although no herds were considered to be Se-deficient based on accepted reference ranges for Se status, a reduction in the odds of being a *Staph. aureus*-positive herd was observed in this study in herds with the highest BTSe concentrations. This latter finding suggests that feeding cows to increase milk Se concentrations in excess of what is currently considered to be adequate, may optimize udder defense against *Staph. aureus* infection. Further study is required to confirm this hypothesis.

4.6   Conclusions

Mean BTSe concentrations appears to be largely adequate in the selected herds. Seasonal variations in pasture Se concentration, or a reduction in Se supplementation when cows go to pasture may cause transient reductions in BTSe in the grazing season, especially in herds from Atlantic Canada. However, Se intake, as reflected in BTSe, does not appear to be a principal determinant of milk yield or BTSCC. Selenium status was much higher, and BTSCC was lower, in the dairy population of our study when compared to experimental herds where an effect of Se on udder health was first described. Nevertheless, a higher BTSe was associated with lower odds of being a *Staph. aureus*-positive herd, possibly as a result of a more robust udder immune response to this mammary pathogen.

4.7 Acknowledgements

4.8 References

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5   BARIUM SELENATE SUPPLEMENTATION AND ITS EFFECT ON INTRAMAMMARY INFECTION IN PASTURE-BASED DAIRY COWS[4]

5.1 Abstract

A significant proportion of cattle receive inadequate dietary Se because of its low content in soils and pastures of various regions of the world. Several economically important diseases in dairy cows, such as mastitis, have been associated with Se deficiency. The objective of this study was to evaluate the effect of a single injection of a long-acting form of Se at drying off on the risk and incidence rate of new intramammary infections, and on milk somatic cell count in the subsequent lactation in pasture-based dairy cows. Forty-nine Chilean Holstein-Friesian cows were fed a diet containing < 0.05 mg Se/kg of ration dry matter. During the dry period, cows were allocated to one of two groups; a supplemented (n = 24) group treated with a single subcutaneous injection of barium selenate (1 mg/kg LW) two months prior to calving, and a control group (n = 25) that remained unsupplemented. Duplicate foremilk samples were aseptically collected within six days after calving, and every two weeks until drying-off for bacteriological culture. Milk samples were also collected monthly for somatic cell count evaluation. Blood samples were collected prior to treatment, and at 30, 90, 180 and 270 days after treatment for analysis of blood glutathione peroxidase activity. Blood glutathione peroxidase activity was higher in supplemented cows 30 days after the injection until the end of the study. The risk and incidence rate of new intramammary infections was not affected by supplementation. A progressive increase in somatic cell count was observed throughout lactation, but there was no effect of supplementation. In conclusion, a one-time injection of barium selenate two months before calving in these pasture-based dairy cows did not affect udder health in the subsequent lactation, indicating that Se basal intake was adequate for preventing subclinical mastitis in pasture-based cows in Southern Chile.

5.2   Introduction

Subclinical and clinical mastitis (CM) are associated with decreased profitability due to decreased milk yield, cost of treatments, early culling, extra labor, discarded milk, and increased rate of cow replacement, making mastitis economically the most important health issue for the dairy industry (Huijps et al., 2008). The immune response mechanisms of the udder effectively deal with contagious or environmental pathogens much more frequently than mastitis occurs (Burton and Erskine, 2003); however, virtually all effectors of the immune response, such as phagocytosis, antibodies, and cell-mediated cytotoxicity are influenced by the nutritional status of the cow and thus nutrition can play an important role in the outcome of IMI (Smith et al., 1997; Ibeagha et al., 2007).

The influence of micronutrients, Se in particular, on the immune response and udder health has been recognized since the early 1980s (Smith et al., 1984; Smith et al., 1985). Providing insufficient Se in dairy cow rations results in a reduced concentration of Se in blood and milk (Maus et al., 1980), downregulation of the expression of many selenoproteins, including glutathione peroxidase (GPx) and thioredoxin reductase, which act as antioxidants with roles in the defense of the mammary gland (Sordillo and Aitken, 2009). Restricted Se intake decreased bactericidal capacity of bovine neutrophils, and inhibited lymphocyte proliferation (Grasso et al., 1990; Ndiweni and Finch, 1995).

North American studies have tended to indicate that low Se intake is associated with a high number of infected quarters at calving and a high SCC (Smith et al., 1984; Smith et al., 1985) whereas studies in pasture-based dairy cows in New Zealand have tended to find no such relationship (Whelan et al., 1992; Grace et al., 1997). The requirement for Se for optimal immune function may be greater than that for other functions (Wichtel, 1998b; NRC, 2001). Conversely, the relatively low incidence of udder health disorders observed in pastoral systems

in regions with low Se intake suggests that immune function is not always impaired in cows moderately deficient in Se, and that environmental and management factors, and intake of other micronutrients such as vitamin E, may modify the requirement for Se for optimal udder immunity (Wichtel, 1998a). Consequently, the literature is unclear regarding the effect of increased Se intake on the defense mechanisms of the mammary gland or on IMI or incidence rate of CM, especially when experimental results derived from North American intensive production systems are compared to those from dairy cows raised in pastoral systems typical of Oceania. There are up to 10-fold differences between countries in recommended Se intakes for dairy cows (NRC, 2001; Grace, 1992), in part because of the inconsistencies mentioned in response to supplementation.

Methods for Se supplementation differ in their cost and practicality for supplementing pasture-based cattle (Wichtel, 1998a). Aqueous solutions for oral use (drenches), intramuscular or subcutaneous injections and ruminal pellets have been used to provide supplemental Se to pastured animals (MacPherson and Chalmers, 1984). A single injection of barium selenate, a source allowed by the European Union to be used in cattle (EMEA, 1999), provides long-duration supplementation in ruminants (Mallinson et al., 1985). An injectable depot product is clearly useful in pasture-based cattle, as only a single administration per lactation is required, ideally before calving, and does not depend on feeding a Se-fortified concentrate ration. Such a method reduces labor, and decreases the risk of transient deficiencies during the grazing season. Nevertheless, the effect of a depot Se product on the incidence of new IMI and on SCC in cows raised in pastoral systems under commercial conditions has not been adequately investigated to date, or only results concerning SCC have been reported (Whelan et al., 1992).

We hypothesized that a single injection of barium selenate before calving may contribute to reduce the risk of having new IMI and SCC in the subsequent lactation. The objective of the study was to determine the effect of a single injection of barium selenate before

calving, on the risk and incidence rate of overall and pathogen-specific new IMI, and on SCC during lactation in pasture-based dairy cows.

5.3 Materials and Methods

All procedures for this trial complied with current regulations for the humane care and treatment of animals, and the Commission for Animal Care and Use of the Universidad Austral de Chile.

5.3.1 Animals and Treatments

The trial was carried out on a commercial dairy farm in southern Chile (39°46' SL-73°13'WL) known to have low Se content in pasture (< 0.05 mg/kg DM). The herd size was, on average, 180 cows with a mean daily milk yield of 19 kg/cow. Previous herd-level analyses of blood GPx activity indicated a mean activity of 40 U/g Hb, reflecting a sub-optimal Se intake (Ceballos et al., 1999). Geometric mean bulk tank SCC in the year before the start of the trial were 259,000 cells/mL.

Forty-nine, clinically healthy primiparous (n = 21) and multiparous (n = 28) pregnant (approximately 7 months of gestation) Chilean Holstein-Friesian cows were selected for this study. Sample size was calculated to detect a difference of 50,000 cells/mL, and a reduction of 40% in the risk of new IMI between groups according to information previously reported (Smith et al., 1985). Cows were weighed before the start of the study, and their weight ranged between 540 and 620 kg. Milk yield per lactation per cow, according to the farm records, was approximately 6,000 kg. Cows were assigned to one of two groups stratified by parity, weight and the predicted date of calving.

Two months before the mean predicted date of calving, cows in the supplemented group

(n = 24) received a single subcutaneous injection of inorganic Se (barium selenate, Deposel,

Young Animal Health Ltd. Wellington, New Zealand) at a dose of 1 mg/kg of live weight. Cows

in the control group (n = 25) received no treatment. The animals did not receive any other

treatment that could affect their Se status.

All cows calved over an 8-week period in the southern hemisphere winter (i.e. mid June

to mid August). Calves were removed from cows within two days after birth. Cows were milked

twice a day in a herringbone style parlor with 8 units and a high milk line. Milk yield was

recorded monthly. Mastitis management included regular monitoring of milking machine

function, post-milking teat disinfection, antibiotic treatment of clinical cases, and antibiotic

treatment of all cows at drying-off. Both groups of cows grazed, as one herd, on pastures

providing less than 0.05 mg Se/kg DM throughout the dry period and lactation. Pastures

comprised of predominantly ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*).

Cows were moved daily onto fresh pasture. To meet nutritional requirements at peak of lactation

(NRC, 2001), the ration was supplemented with silage made from the same pasture, and

commercial concentrate (2.9 Mcal/kg and 15% of crude protein DM basis; Cosetan Vaca

Lechera, Biomaster-IANSA, Temuco, Chile). The concentrate offered per cow was, on average,

0.25 kg per kg of milk yield. Additionally a Se-free mineral mix (Vetersal, Veterquimica,

Santiago, Chile) was fed at a rate of 200 g/cow/day. Animals did not receive any other treatment

that could affect their Se status, and water was offered ad libitum.

5.3.2   Sampling and Data Collection

The farmer was trained to collect milk samples aseptically from those quarters that had

physical signs of CM (any visual abnormality of milk or udder, with or without systemic signs of

disease), at any stage of the study. In addition, a physical examination of the mammary gland

and milk characteristics was conducted within 6 days after calving and whenever a milk sample was collected to detect individual quarters that were inflamed (hard, swollen or hot) due to CM. The first streams of milk were squirted into a dark cup to detect any abnormality such as discoloration, flakes, clots or wateriness.

Duplicate foremilk samples (15 mL) for bacteriological analysis were aseptically collected from every quarter by an experienced veterinarian and technicians in the first week after calving and thereafter every 14 days until the end of lactation. For each quarter, the first stream of milk was discarded, and then the teat end was wiped to remove gross contamination, and finally disinfected with a cotton swab soaked in 70% alcohol. Immediately after collection, samples were refrigerated at 5°C for transportation to the laboratory. Additionally, composite milk samples (30 mL) from each cow were collected monthly for SCC determination using potassium dichromate as preservative, kept refrigerated and analyzed within 48 hours. Coccygeal venous blood samples were collected from control and treated cows prior to treatment and at 30, 90, 180, and 270 days after the treatment date into heparinized vials to determine the activity of GPx.

5.3.3 Laboratory Analyses

Udder health status was established through clinical and bacteriological examinations of the mammary gland. Bacteriological examination was performed according to the standards of the NMC (Oliver et al., 2004). A volume of 0.025 mL of milk was streaked onto one quadrant of an esculin blood (sheep) agar plate, keeping it at room temperature for 30 min, and then aerobically incubated at 37°C for 24 to 48 hours. For each plate, the number of colony-forming units of each bacterial species was counted, and a single well-isolated colony subcultured for bacterial species identification. Pathogens were initially classified according to colony morphologic features, hemolytic characteristics, and Gram stain reaction.

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Bacterial species were further identified following conventional procedures. Species presumptively identified as staphylococci were tested by the tube coagulase method and deoxyribonuclease degradation test in DNase agar plates. *Staphylococcus aureus* was identified by a positive coagulase reaction and a positive DNase test. Coagulase-negative staphylococci were identified by a negative coagulase reaction, and a negative DNase test (Devriese et al., 1985). Isolates presumptively identified as streptococci were tested for Christie, Atkins, Munch-Petersen test reaction and biochemical properties (SVA Strep, SVA, Uppsala, Sweden). Gram-negative isolates were classified as *Escherichia coli* when positive for the production of ß-D-glucuronidase and indole (PI Test, SVA, Uppsala, Sweden). Strains that were negative were further tested by the API20E identification system (bioMérieux SA, Marcy l'Etoile, France), which permits a rapid species identification of *Enterobacteriaceae* and other Gram-negative organisms (Oliver et al., 2004).

Somatic cell count was determined using a Combifoss 5300 cell counter (Foss Electric, Hillerød, Denmark). To approximate the normal distribution, data were expressed as natural logarithm of SCC in thousands/mL (LnSCC; 1,000 cells/mL).

Selenium in pasture and concentrate was evaluated at the beginning of the study, and twice during the study period by inductively coupled plasma mass spectroscopy (Hill Laboratories, Hamilton, New Zealand). The content of Se was measured in milligrams per kilogram of dry matter.

The effective amount of Se delivered to the cows, on a per cow per day basis, was estimated by multiplying the average DMI of pasture, silage and concentrates by their average Se concentration. Mean Se concentration was 0.035 mg/kg DM and 0.1 mg/kg DM for pasture and concentrate, respectively. The effective amount of Se delivered to the cows via the depot injection of barium selenate was not evaluated. However, an early trial conducted in the United Kingdom indicated that the release of Se from the site of injection is not constant over time,

estimating the payout of Se from a single depot injection to be, on average, 0.8 mg Se/day (95%

CI: 0.28, 1.27 mg Se/day) in heifers (Mallinson et al., 1985). Authors arrived at this estimate by

measuring the residual barium selenate at the site of injection 17 weeks after treatment.

The Se intake recommended, on a per cow per day basis, for intensive dairy systems

(NRC, 2001) and for extensive pasture-based systems in New Zealand (Grace, 1992; Wichtel,

1998a) was extrapolated by multiplying the total estimated DMI by the recommended diet Se

concentration. Based on these assumptions, recommended daily Se intake was estimated at 4.7

and 0.6 mg Se/cow/day for the intensive and extensive management systems, respectively.

The Se status was evaluated through the blood GPx activity by a kinetic method (Ransel,

Randox Lab, Crumlin, UK). GPx is a Se-containing enzyme currently used as a biomarker of Se

status. The method used approximates an early methodology (Paglia and Valentine, 1967) with

slight modifications (Davidson et al., 1990). Briefly, hemoglobin in fresh blood was measured

by the cyanomethemoglobin method prior to GPx analysis. Readings of blood GPx activity were

made in a Cobas Mira chemistry analyzer (ROCHE Diagnostics GmbH, Mannheim, Germany).

The enzyme activity is proportional to the decrease in absorption at 340 nm after the oxidation of

NADPH into NADP (Davidson et al., 1990). The enzyme activity was measured in units per

gram of hemoglobin, considering an activity below 100 U/g Hb as indicator of suboptimal Se

status (Ceballos et al., 1999).

5.3.4   Definition of Intramammary Infection

A culture was considered positive when ≥ 1 cfu/0.025 mL (equivalent to ≥ 40 cfu/mL) of

a pathogen was obtained. A quarter was considered to have an IMI in the first week after calving

when the same pathogen was cultured from both duplicate samples, while after calving an IMI

was considered when the quarter was culture-positive for the same pathogen in two out of three

consecutive samples, a slight modification of the rule used by Zadoks et al. (2001). During

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lactation, a quarter was considered to have a new IMI when a pathogen was cultured in any of the following cases:

i.   A culture-negative quarter in the first week after calving that met the IMI criteria after week 3.

ii.   An infected quarter, past week 3, that was negative for the previous two samples.

iii.   An infected quarter, past week 3, that met the IMI criteria but for a different pathogen.

A previously infected quarter was considered recovered from IMI for a bacterial species if none of the above definitions were met and the sample was free of the pathogen on, at least, two consecutive tests (Zadoks et al., 2001, with slight modifications). Samples containing more than two bacterial species were considered contaminated, and were not informative of IMI status. The duration of an IMI was expressed on the basis of the number of calendar days that the quarter was positive. Thus, a positive quarter on one test was positive for 14 days (Smith et al., 1984). The number of recovered (i.e. cured) quarters, according to the previous IMI definition, was also recorded.

5.3.5 Statistical Analysis

The overall and pathogen-specific incidence risk of new IMI were calculated as the probability that an individual quarter had a new IMI during lactation. Quarter of the cow was the unit of interest, and only quarters free of infection with the pathogen of interest at calving were included in the analysis. The effect of Se supplementation on this variable was evaluated using a logistic regression model with cow random effects (Dohoo et al., 2009). The resulting model was as follows:

$$\text{logit } (p_{ij}) = \beta_0 + \beta_1 \text{treatment}_j + u_j \tag{1}$$

where p_{ij} is the probability of having a new IMI or a pathogen-specific new IMI in the ith quarter of the jth cow. β_0 is the constant; β_1 is the regression coefficient for treatment effect, and u is the cow random effect. The coefficients of the regression were expressed as odds ratio.

The overall and pathogen-specific incidence rate were defined as the number of new quarter infections per quarter-time period at risk, with time period being a 14-d interval. Consequently, quarter-time at risk for a new pathogen-specific IMI was calculated as the total time (i.e. number of 14-d intervals) excluding those intervals with an ongoing IMI. The unit of interest for the analysis of incidence rate was the quarter of the cow. The effect of Se supplementation was evaluated using a Poisson regression model, accounting for cow as random variable with the number of new pathogen-specific IMI as the outcome, and quarter-time at risk as the offset. The resulting model was as follows:

$$\text{Ln } [E(I_{ij})] = \beta_0 + \beta_{1\,treatment_j} + u_j \qquad [2]$$

where Ln $[E(I_{ij})]$ is the natural logarithm of the expected value of the overall and pathogen-specific incidence rate of IMI (I = number of new infections/time periods at risk). β_0 is the constant; β_1 is the regression coefficient for treatment effect, and u is the cow random effect. The coefficients of the regression were expressed as incidence rate ratio. All analyses were carried out using the 'xtlogit' and 'xtpoisson' commands of the statistical software STATA version 11.0 (Stata Corp, College Station, TX, USA).

The repeated measurements of LnSCC and GPx (days 0-270 post treatment) on each cow were analyzed separately using linear mixed models (Dohoo et al., 2009). Different within-cow correlation structures were evaluated. Results from first order autoregressive (AR 1), first order autoregressive moving average (ARMA 1,1) and Toeplitz were compared by likelihood-

ratio tests. The effects of supplementation and time (month for LnSCC, and days for GPx) as well as their interaction were also evaluated. The resulting model for LnSCC or blood GPx was as follows:

$$y_{ij} = \mu + \alpha_{\text{treatment}(i)} + \beta_j + (\alpha\beta)_{\text{treatment}(i)j} + \varepsilon_{ij}$$ [3]

where y_{ij} corresponds to LnSCC or blood GPx activity in the ith cow at time j. μ is the overall mean; α_i is the treatment effect; β_j represents the effect of time (month for LnSCC or days for GPx); $(\alpha\beta)_{ij}$ is the interaction between treatment and time.

The models were evaluated by examining the standardized residuals, and transformations of the outcome were explored whenever the model assumptions were not fully met. Analyses were carried out using the MIXED procedure of the statistical software SAS version 9.1.3 (SAS Institute Inc, Cary, NC, USA).

5.4 Results

The mean period from the start of the trial to calving was 62 days (95% CI: 57, 66). One cow in the control group died 10 weeks before drying-off, but data were kept for the statistical analysis, as the cause of death was not related to udder health. All milk samples from one of the supplemented cows collected over the 10-mo period were not suitable for SCC analysis because they were clotted.

Mean and standard deviation of parity in the supplemented and unsupplemented cows was 2.5 ± 1.4 and 2.1 ± 1.4, respectively, ranging from 1 to 5. Daily milk yield was 19.0 ± 0.65 kg/day and 20.6 ± 0.62 kg/day for supplemented and unsupplemented cows. Analysis by

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contrasts in a linear model showed that the difference between supplemented cows and unsupplemented controls was not significant ($P = 0.09$).

5.4.1 Blood Glutathione Peroxidase Activity

The model validation for the analysis of blood GPx did not reveal any non-compliance with model assumptions. The optimal correlation structure for the within-cow dependence activity was Toeplitz, with correlations of 0.35; 0.07; -0.48; and -0.59. The statistical analysis showed a strongly significant effect of the interaction between supplementation with barium selenate and days after the treatment ($P < 0.001$). Blood GPx activity was significantly higher in the supplemented group from 30 days after the injection with barium selenate, which was the first sampling after the injection (Figure 5.1). The highest activity was observed 90 days after the treatment, barium selenate-treated heifers had a mean blood GPx activity of 289 U/g Hb compared to 52 U/g Hb in the unsupplemented controls (Figure 5.1).

Selenium delivered to the cows was compared on a per cow per day basis to the recommended daily intakes. The basal Se intake in both groups of cows was approximately 0.9 mg Se/cow/day, which corresponded to approximately 20% of the amount recommended by NRC. However, the basal Se intake was 160% of the amount recommended for dairy cows raised in pastoral systems (Grace, 1992).

5.4.2 Intramammary Infection and Clinical Mastitis

At calving, 17 of 196 quarters (8.7%) had an IMI. During lactation, a total of 3936 milk samples were collected for bacteriologic analysis of which 3141 (79.8%) were culture-negative, and 7 (0.2%) were contaminated. Pathogens were isolated in 788 (20.0%) milk samples with C. bovis (53.3%), *Staph. aureus* (21.4%), and coagulase-negative staphylococci (19.4%) being the most common isolated pathogens.
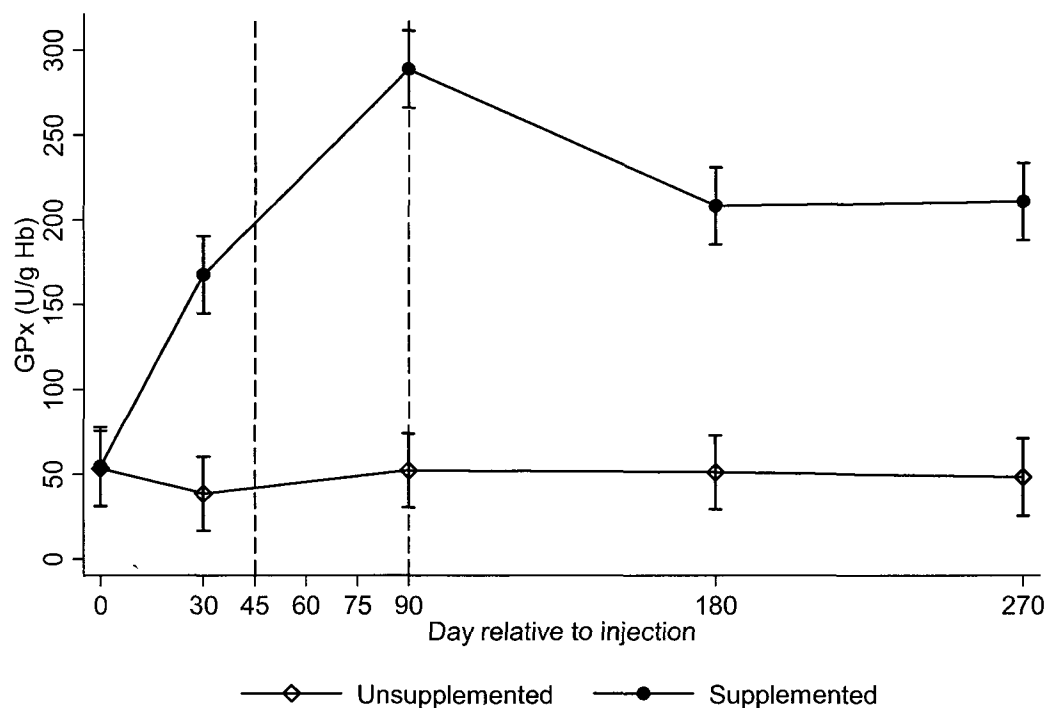
Figure 5.1. Least square means and SE of activity of glutathione peroxidase (GPx) in blood in pasture-based dairy cows, which were either supplemented with barium selenate before calving (n = 24), or left unsupplemented (n = 25). Dashed lines indicate beginning and end of the calving period in relation to the time of treatment with barium selenate. Differences between groups were statistically significant from day 30 onwards ($P < 0.001$).

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A total of 100 new IMI were found in both groups of cows, 58 in supplemented cows, and 42 in unsupplemented controls. The duration of IMI ranged from 14 to 165 days in the supplemented cows, and ranged from 32 to 154 days in unsupplemented cows. A total of 165 quarters were cured (Table 5.1). The proportion of cured quarters in supplemented cows (24%) was similar to the proportion observed in the unsupplemented group (15%) ($P = 0.11$).

The odds of new IMI during lactation, although numerically higher in the supplemented group, was not different between supplemented and unsupplemented cows (Table 5.2). The odds of pathogen-specific IMI was similar between groups. Parity did not have any effect on the overall odds of new IMI ($P = 0.51$), or the odds of pathogen-specific new IMI (data not shown) during lactation.

The crude overall incidence rate of new IMI during lactation was 0.011 and 0.008 new IMI per 14-day interval at risk for supplemented and unsupplemented cows, respectively. No differences in the overall incidence rate ratio, and the pathogen-specific incidence rate ratio were found (Table 5.3). Parity of the cow did not affect the incidence rate ratio of new IMI during lactation ($P = 0.52$).

At calving, no cases of CM were detected in the supplemented cows, while one case was found in the unsupplemented group. During lactation, 18 cases were detected, 8 of them in the supplemented cows, and 10 in the control cows. However, the odds of getting CM during lactation were not associated with supplementation (odds ratio: 0.84, 95% CI: 0.19, 3.61) or parity ($P = 0.95$).

Table 5.1. Number of quarters with an intramammary infection at calving, new infections and their duration in quarters of lactating pasture-based dairy cows injected with barium selenate before calving (n = 24), compared to unsupplemented control cows (n = 25).

Item	Supplemented					Unsupplemented				
	C^1	L^2	Duration[3]		Cure[4]	C^1	L^2	Duration[3]		Cure[4]
Staphylococcus aureus	5	9	165	(21, 259)	4	3	10	42	(28, 182)	5
Streptococcus dysgalactiae	0	1	14		1	0	1	154		1
Streptococcus uberis	0	2	70		0	0	1	42		1
Streptococcus bovis	1	2	35	(28, 42)	2	1	1	32	(28, 35)	1
Streptococcus spp	0	5	28	(14, 42)	5	0	1	84		1
Escherichia coli	0	1	14		1	0	0			0
Coagulase-negative staphylococci	4	11	53	(28, 182)	8	3	7	35	(14, 238)	4
Corynebacterium bovis	0	27	125	(70, 182)	11	0	21	140	(84, 238)	4

[1]Number of infections at calving.
[2]Number of new IMI during lactation.
[3]Duration of the infection in the quarter (median, and lower and upper quartiles).
[4]Number of cured quarters.

Table 5.2. Effect of selenium supplementation on the overall odds and pathogen-specific odds of new intramammary infection in pasture-based dairy cows injected with barium selenate before calving (n = 24), compared to unsupplemented control cows (n = 25).

Item	OR[1]	95% CI[2]	P-value
Overall	2.31	0.69, 7.80	0.17
Pathogens			
Staphylococcus aureus	0.74	0.14, 3.95	0.73
Streptococcus dysgalactiae	1.04	0.06, 16.88	0.98
Streptococcus uberis	1.60	0.02, 129.20	0.83
Streptococcus bovis	2.11	0.19, 23.60	0.55
Streptococcus spp.	5.90	0.54, 64.40	0.14
Coagulase-negative staphylococci	1.73	0.62, 4.82	0.30
Corynebacterium bovis	1.96	0.50, 7.61	0.33

[1]Odds ratio.
[2]95% confidence interval.

Table 5.3. Effect of selenium supplementation on the incidence rate of new intramammary infection in pasture-based dairy cows injected with barium selenate before calving (n = 24), compared to unsupplemented control cows (n = 25).

Item	IRR[1]	95% CI[2]	P-value
Overall	1.36	0.83, 2.22	0.22
Pathogens			
Staphylococcus aureus	1.97	0.38, 10.20	0.42
Streptococcus dysgalactiae	1.02	0.06, 16.26	0.99
Streptococcus uberis	1.34	0.03, 66.73	0.88
Streptococcus bovis	2.05	0.18, 22.61	0.56
Streptococcus spp.	6.42	0.59, 70.20	0.13
Coagulase-negative staphylococci	1.96	0.65, 5.93	0.23
Corynebacterium bovis	1.73	0.62, 4.86	0.30

[1]Incidence rate ratio.
[2]95% confidence interval.

5.4.3 Somatic Cell Count

The optimal correlation structure for the within-cow dependence was autoregressive-moving average (ARMA 1,1) for the analysis of LnSCC, with an estimated baseline correlation of 0.97 (SE = 0.01), which decreased by a factor of 0.73 (SE = 0.04) for each month of separation. Therefore, an estimated correlation 1 month apart was 0.97*0.73=0.71.

Somatic cell count increased steadily from the first month of lactation to drying-off in both groups ($P < 0.01$; Figure 5.2). Geometric mean SCC in the first month of lactation was 29,000 cells/mL compared with 148,000 cells/mL in the last sample before drying-off.

Supplementation did not affect SCC nor was there an interaction between treatment and time. Geometric mean SCC was 76,000 and 87,000 cells/mL for supplemented and unsupplemented cows, respectively ($P = 0.68$). Neither parity ($P = 0.72$) nor its interaction with treatment was associated with LnSCC ($P = 0.52$).

5.5 Discussion

Activity of GPx was higher in supplemented than in unsupplemented cows by the time of the first post-treatment sample (30 days after the treatment), remaining higher in treated cows for the rest of the study. The blood GPx activity has much laboratory-to-laboratory variation, therefore our results were compared to reference values obtained using the same method, reflecting an adequate Se status based on Chilean reference ranges (Ceballos et al., 1999). However, this difference was not translated into enhanced udder resistance to pathogens. No differences between groups for the incidence rate of new IMI were observed. Somatic cell count significantly increased in both groups until the end of the trial, not being affected by Se supplementation.
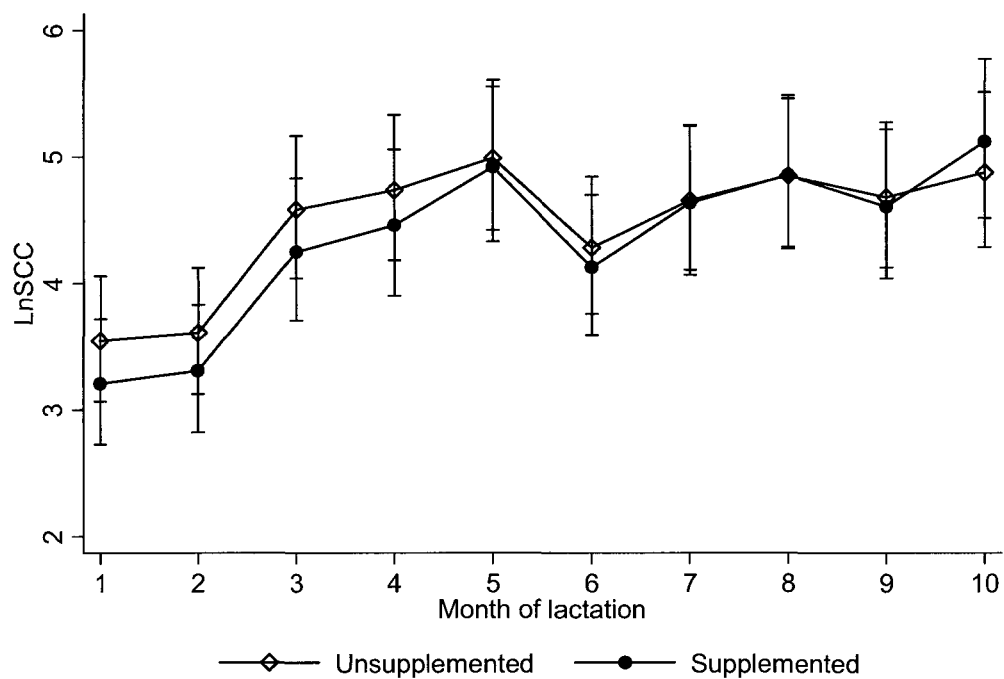
Figure 5.2. Least square means and SE of natural logarithm of somatic cell counts (LnSCC; 1,000 cells/mL) in milk of pasture-based dairy cows, which were either supplemented with barium selenate before calving (n = 23), or left unsupplemented (n = 25). Differences between the supplementation groups were not statistically significant; the effect of time was significant ($P < 0.001$).

Blood activity of GPx varies with Se intake. Therefore its evaluation is useful as a biomarker of Se status, giving a good indication of the incorporation of Se into functional selenoproteins (Arthur, 1999; Grace et al., 2001). In our study, basal Se intake of cows was lower than the 4.7 mg/cow/day recommended by NRC (2001). However, the single injection of barium selenate before calving contributed an undetermined daily amount of supplemental Se that was enough to cause a substantial difference in the blood GPx activity between groups. These results concur with other studies indicating a steady rise in GPx activity after the injection of barium selenate (Grace et al., 2001). There is a delay (~ 45 d) between the supplementation and the rise in blood GPx activity, which results from the incorporation of Se into the enzyme during erythropoiesis (Grace et al., 2001). In this trial, the long-acting Se preparation increased blood GPx activity up to 270 days after treatment.

Few trials have examined the dynamics of IMI in unsupplemented and Se-supplemented cows. Of those that appear in the peer-reviewed literature, all have been in intensively managed confined herds and, in all, a negative association between Se status and the incidence of IMI was found (Smith et al., 1985; Malbe et al., 1995). Selenium supplementation was highly protective in one study, translating into fewer quarters infected with coagulase-positive staphylococci (non-*Staph. aureus*) at calving, fewer cases of CM, shorter duration of infections, and lower milk SCC in Se-supplemented primiparous cows; however, no differences in the rate of IMI were observed further during lactation (Smith et al., 1985). Malbe et al. (1995) reported a reduction of both the prevalence of IMI and high SCC when mid-lactation cows in intensive systems were fed with Se either inorganic or organic (0.2 mg Se/kg DM). Even though the cows of our study had a lower basal Se intake, we did not observe any effect of Se supplementation on the risk of IMI during lactation, which concurs with the results reported by Smith et al. (1985). We are not aware of reports on the dynamics of IMI comparing Se-supplemented and unsupplemented cows under pastoral systems comparable to our study.

In the present study, geometric mean SCC increased steadily from the beginning to the end of the trial, similar to what was described previously (Schepers et al., 1997). Within lactation, geometric mean SCC of uninfected cows is affected by the amount of milk (Schepers et al., 1997); the increasing mean SCC with increasing DIM will therefore partly be the result of a decrease in milk production during lactation. Additionally, SCC at the end of lactation will also be affected by a higher prevalence of IMI, which increases with the stage of lactation (Weller et al., 1992; Schepers et al., 1997).

Observational studies and trials in the United States (Smith et al., 1985; Erskine et al., 1987; Weiss et al., 1990) and Estonia (Malbe et al., 1995) suggest a negative relationship between plasma Se or blood GPx and SCC. Our results did not support those findings, agreeing with other studies that found no association between either an adequate Se status (Grace et al., 1997; Nyman et al., 2008) or Se supplementation (Coe et al., 1993; Bourne et al., 2008) and SCC. In New Zealand studies with pasture-based dairy cows supplemented with barium selenate (Whelan et al., 1992) or intra-ruminal pellets containing Se (Wichtel et al., 1994), researchers did not observe a reduction of SCC in the milk of supplemented cows, suggesting that giving supplemental Se beyond the estimated requirement may not offer further advantage to reduce the risk of new IMI or SCC under these management conditions.

Discrepancies between the results presented here and those of researchers in other regions, particularly in the United States, may be explained by a variety of non-nutritional and nutritional factors other than Se intake. First, a non-nutritional factor might be the study design, as our design had sufficient power to detect a difference of 40,000 cells/mL and a reduction of 40% in the risk of new IMI between groups, but the obtained data did not reflect such differences between groups. A second reason might be related to the presence of other dietary factors that may interact with Se requirements, such as the intake of vitamin E (NRC, 2001). Most cows in pastoral dairy systems will experience sufficient intake of vitamin E when fresh

forage is fed, and a higher vitamin E intake appears to have a sparing effect on Se (Wichtel, 1998b) but the relationship has not been quantified (NRC, 2001) and may be affected by the consumption of fatty acids in the ration (Wichtel et al., 1996). Clearly, when Se intake is lower than recommended values, vitamin E influences the incidence and severity of mastitis in dairy herds (Weiss et al., 1997).

A third possible reason is related to the differences in the pathogen challenge between intensive and pastoral systems. The pathogen challenge during this study might have been insufficient (either type or degree) as compared to the challenges in other studies, to demonstrate a difference between groups in resistance to IMI. Early studies on the effect of Se supplementation and udder health started with a higher prevalence of IMI at calving as compared with the prevalence observed in our cows, and were focused on the effect of Se supplementation on the incidence of environmental mastitis (Smith et al., 1984; Smith et al., 1985), as confinement favors IMI by environmental pathogens. The number of new IMI caused by environmental pathogens was low in our study. Finally, another concern in the current study design may be related to the fact that supplemented and unsupplemented cows were pastured and milked together; if one of the groups has an increased incidence of IMI, this might influence the incidence of the other group. However, if a difference in udder health would have been found, and the cows would not have been pastured and milked together, this difference could very well have been the result of other factors that were different between the two groups.

Whether NRC (2001) recommendations for Se intake are applicable under Southern Chilean conditions is uncertain. However, under the conditions of our experiment, clearly this inadequacy in Se intake did not appear to be a significant factor in the susceptibility to IMI, and cows on pasture in Southern Chile probably have Se requirements, at least for optimal udder immunity, that are no higher than the actual intakes of the unsupplemented cows in our study.

5.6   Conclusions

Supplementation using a long-duration injection of barium selenate in pasture-based dairy cows increased blood GPx activity. However, this difference in Se status was not translated into differences in the risk of new IMI or SCC pattern. Discrepancies with previously published results on the effect of Se supplementation on udder health may be related to variations in the requirement of Se for dairy cows in pastoral systems compared to the current recommendation for cows in intensive systems. Under the conditions of this study, Se supplementation before calving did not result in improved measures of udder health in the subsequent lactation, indicating that Se basal intake seemed to be adequate for preventing subclinical mastitis.

5.7   Acknowledgements

5.8   References

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6   THE EFFECT OF SELENIUM SUPPLEMENTATION BEFORE CALVING ON EARLY-LACTATION UDDER HEALTH IN PASTURED DAIRY HEIFERS[5]

6.1 Abstract

Selenium (Se) deficiency has been associated with lowered resistance to mastitis in dairy cattle. However, there is little published data on the effect of Se supplementation before calving on udder health of pastured dairy heifers. Further, the relative efficacy of injectable barium selenate and oral organic Se for improving udder health in cows has not previously been tested. The objectives of this study were to determine the effects of pre-calving Se supplementation, and type of supplementation, on the blood activity of glutathione peroxidase and measures of udder health immediately after calving and during the first month of lactation in pastured dairy heifers. One hundred and forty pregnant Chilean Holstein-Friesian heifers were fed a basal diet containing, on average, 0.15 mg Se/kg of dry matter. One month before predicted calving, heifers were allocated to one of three groups. Group 1 (n = 49) received no supplementary Se, group 2 (n = 46) received a single subcutaneous injection of Se (1 mg/kg of live weight, as barium selenate), and group 3 (n = 45) was fed Se yeast (3 mg/heifer/d until calving). Heifers supplemented with barium selenate had a higher glutathione peroxidase activity from 14 d in milk onwards. Selenium supplementation, irrespective of source, tended to reduce the prevalence of intramammary infection (IMI), and decrease the prevalence of quarters with high somatic cell count (SCC) at calving. Selenium supplementation did not result in a reduction of the incidence of new IMI, clinical mastitis or in SCC during the balance of the first month of lactation. However, no incident cases of clinical mastitis were observed in heifers injected with barium selenate in the first month of lactation. In conclusion, Se supplementation beginning one month before calving in first lactation heifers at pasture decreased the prevalence of IMI and SCC shortly after calving irrespective of the source of Se.

6.2   Introduction

Heifers should be at low risk for IMI at first calving, as they produce less milk, have not

experienced multiple daily milkings, have less exposure to contagious pathogens, and are less

likely to be in contact with the environment (Fox, 2009). However, a wide variation in the

immediate postpartum prevalence of IMI has been reported in quarters of first calving heifers,

ranging from 12 to over 57%, with coagulase-negative staphylococci (CNS) being the most

prevalent pathogens associated with IMI (Compton et al., 2007; Fox, 2009; Piepers et al., 2010).

The incidence of clinical mastitis (CM) may reach 35% during the peripartum period (Fox,

2009). The peak incidence may be higher than that found in multiparous cows, with > 30% cases

in first lactation heifers occurring during the first 14 DIM (Barkema et al., 1998; Olde Riekerink

et al., 2008; Sampimon et al., 2009). Yet, the prevalence and incidence of IMI and CM in

peripartum heifers varies greatly between management systems (Fox, 2009), suggesting that

management interventions at farm level may be useful in reducing the risk of these infections.

Many important risk factors are associated with subclinical and CM in heifers. Links

between nutrition and mastitis control, in particular the micronutrients Se and vitamin E, have

been studied extensively (Smith et al., 1997; Heinrichs et al., 2009). Providing insufficient Se in

dairy cow rations results in a reduced concentration of Se in blood and milk (Maus et al., 1980),

lower blood glutathione peroxidase (GPx) activity and other antioxidants with roles in the

defense of the mammary gland (Sordillo et al., 2007), decreased bactericidal capacity of

neutrophils, and inhibited lymphocyte proliferation (Grasso et al., 1990) which, acting together,

are believed to impair the immune response of the udder to pathogens.

In a North American study, supplementation of heifers with Se resulted in a lower

prevalence of IMI at calving, lower incidence of CM, shorter duration of IMI, and lower SCC

(Smith et al., 1985). The estimated equivalent Se intake for those heifers was beyond the current

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recommendation for dairy cows, which is 0.3 mg/kg dry matter (DM) (NRC, 2001). However, studies in pasture-based dairy cows in New Zealand have found no relationship between Se supplementation and udder health, as measured by SCC (Whelan et al., 1992; Wichtel et al., 1994; Grace et al., 1997). In support of these findings, a recent trial conducted in southern Chile, where a long-acting Se supplement was administered at drying off to moderately Se deficient pastured based dairy cows, resulted in a sustained increase in blood GPx activity. However, no change was found in the incidence of new IMI or SCC during the subsequent lactation (Chapter 5), suggesting that the Se requirement relative to IMI in dairy cows under pastoral systems may be lower than the current NRC (2001) recommendation for cows raised in intensive systems. These findings suggest that the response to Se supplementation in measures of udder health may differ depending on the system under which the experimental animals are managed.

Uniform, reliable delivery of mineral supplements to cattle is a challenge under pastoral conditions; free-choice feeding of mineral supplements often results in intake that is inconsistent within and between animals in the herd. A single injection of a slow-release form of Se (e.g. barium selenate) provides, not only a long duration of effect, but also an appropriate daily delivery Se, suitable for correcting deficiency in ruminants under most pastoral systems (Mallinson et al., 1985). This form is particularly useful in pastured cattle because it needs to be administered only once per lactation, ideally before calving, and does not depend on a system for feeding a Se-fortified concentrate ration. Selenium can be also supplemented to cattle diets incorporating organic forms in concentrates. Cattle fed Se yeast usually have a higher blood Se concentration and higher GPx activity in blood (Malbe et al., 1995) and milk (Chapter 2) than do those supplemented with inorganic sources. However, most studies that found a beneficial role of Se on measures of udder health used Se as sodium selenate/selenite. It is unknown whether the typically higher GPx activity (i.e. improved Se status) when cows are injected with barium selenate or fed Se yeast reflects an improved udder health compared to supplementation using

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traditional inorganic sources (e.g. sodium selenite). In addition, clear guidelines on optimal Se supplementation practices for grazing heifers, in particular to enhance the udder immune response, are not available to herd managers at present.

Our hypothesis was that Se supplementation before calving may be associated with a lower risk of new IMI and SCC around calving in heifers grazing low-Se pastures. A former Chilean trial indicated no effect of Se supplementation on measures of udder health in multiparous cows during lactation. However, heifers in pastoral systems may be at a higher risk of Se inadequacy compared to multiparous cows (Ceballos et al., 1998). The objectives of this study were to determine the effects of pre-calving Se supplementation, and type of supplementation (injectable barium selenate vs. oral Se yeast), on the blood activity of GPx and measures of udder health immediately after calving and during the first month of lactation in pastured dairy heifers.

6.3   Material and Methods

All procedures for this trial complied with current regulations for the humane care and treatment of animals, and the Commission for Animal Care and Use of the Universidad Austral de Chile.

6.3.1   Animals and Treatments

The trial was carried out on four commercial dairy farms in the south of Chile (39°46' SL-73°13' WL) where prior tests had revealed marginal Se concentration in pastures (~ 0.1 mg/kg DM), and herd-level analyses of blood GPx activity had indicated a mean ≤ 100 U/g Hb,

consistent with sub-optimal Se intake using a published reference range for blood GPx in Chilean cattle (Ceballos et al., 1999).

One hundred and forty pregnant Chilean Holstein-Friesian heifers that were predicted to calve approximately 2 months after the day of their treatment were selected for the study. Heifers on each farm were randomly allocated to one of three treatments by a systematic random process. Briefly, the first heifer being run through a chute was assigned to the unsupplemented control Group 1, the second to Group 2, and the third heifer to Group 3, repeating the sequence with the subsequent animals. Group 1 (n = 49) received no supplementary Se; Se consumption corresponded to the basal intake for the herd. Heifers in Group 2 (n = 46) received a single subcutaneous injection of a 1 mg/kg of live weight Se as barium selenate (Deposel, Young Animal Health Ltd. Wellington, New Zealand). Heifers in Group 3 (n = 45) were fed 3 mg/heifer/d Se as Se yeast (Sel-Plex, Alltech, Nicholasville, KY) carried in 250 g of wheat hulls, from the first day of treatment until calving. Unsupplemented and barium selenate-treated heifers were maintained on pasture under similar farm management conditions, and fed the same ration for close up and lactation (Table 6.1). Heifers of Group 3 were separated for feeding the wheat hulls.

Heifers calved within an 11-week period in the southern hemisphere winter and spring (i.e. mid July to mid October of 2007). Calves were removed from their dams within one day after birth. Heifers were milked twice daily starting one day after calving. Milk yield was recorded at the end of the 28-day study period. Mastitis management included regular monitoring of milking machine function, post-milking teat disinfection, antibiotic treatment of clinical cases, and antibiotic treatment of all cows at drying-off. Before and after calving, the animals comprising the three experimental groups (within herd) were separated from each other, and grazed perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The same pastures were used to make silages and hays on each farm. Heifers were moved daily onto fresh

pasture, and were fed with a close up diet consisting of supplemental silage or hay, commercial concentrates and mineral mixes for the last three weeks of gestation. After calving, animals were still on pasture and were supplemented with silage, concentrates and mineral mixes (Table 6.1). The daily amount of concentrate offered per heifer was, on average, 1 kg/4 kg of milk yield. Mineral mixes were offered at a rate of 200-400 g/head/day. Both supplements, concentrates and mineral mixes, contained Se as sodium selenite. Water was offered ad libitum. Animals did not receive any other treatment that could affect their Se status.

Daily DMI was, on average, 9.0 and 15.5 kg per day for the close up and lactation diets, respectively. The effective amount of basal Se delivered to the heifers via the basal diet, on a per day basis, was estimated as the average of the Se concentration of the components, weighted by the average DMI of each component (Table 6.1). The basal Se intake was approximately 50% of the recommended amount for close-up, and fresh heifer rations (NRC, 2001). The effective amount of Se delivered to the heifers via the depot injection of barium selenate was estimated to be, on average, 0.8 mg Se/day (95% CI: 0.28, 1.27 mg Se/day) in heifers (Mallinson et al., 1985). Mallinson et al. (1985) arrived at this estimate by measuring the residual barium selenate at the site of injection 17 weeks after treatment; however, this is likely to be an underestimate of the daily payout in the weeks immediately after injection.

6.3.2 Sampling and Data Collection

Milkers on each farm were trained to collect milk samples aseptically from quarters that had physical signs of CM (any visual abnormality of milk or udder, with or without systemic signs of disease). The first streams of milk were squirted onto a dark cup to detect any abnormality such as discoloration, flakes, clots or wateriness. This physical examination of the udder and milk characteristics was conducted at calving, and also at every milking, to detect individual quarters affected by CM.

Colostrum and milk samples (15 mL) for bacteriological analysis were aseptically collected from every quarter by an experienced veterinarian or technicians on the day of calving, and thereafter at 1, 7, 14, 21 and 28 DIM.

For each quarter, the first streams of milk were discarded; the teat end was scrubbed to remove gross contamination, and finally disinfected with a cotton swab soaked in 70% isopropyl alcohol. Immediately after collection, samples were refrigerated at 5°C for transportation to the laboratory. Quarter colostrum and milk samples (~ 30 mL) were also collected in vials with potassium dichromate for SCC determination. Samples were kept refrigerated and analyzed within 24 hours.

A batch of 1053 (31%) milk samples was lost after a fire destroyed the laboratory of microbiology of the Faculty of Sciences of the Universidad Austral de Chile (Appendix 4).

Coccygeal venous blood samples were collected from control and treated heifers at the beginning of treatment, 14 and 28 DIM into heparinized vials to determine the blood GPx activity.

6.3.3   Laboratory Analyses

Bacteriological examination was performed according to the standards of the National Mastitis Council (Oliver et al., 2004). An esculin blood (sheep) agar plate was divided and 0.05 mL of milk were streaked onto one half, keeping it at room temperature for 30 min and then aerobically incubated at 37°C for 24 to 48 hours. For each half of the plate, the number of colonies is expressed as cfu/0.05 mL. A single well-isolated colony was subcultured for bacterial species identification according to colony morphologic features, hemolytic characteristics, and Gram stain reaction.

Table 6.1. Average composition of the close up and lactation diets (% of ration dry matter), mean and range of selenium concentration of the components, total daily Se intake (mg/heifer/d), and suggested Se intake for each physiological status.

Item	Close up	Lactation	Se (mg/kg DM)
Ingredient (% of DM):			
Grass	52.3	22.4	0.10 (0.02, 0.16)
Grass silage or hay[1]	14.5	33.0	0.09 (0.02, 0.14)
Concentrate[2]	32.2	43.3	0.10 (0.05, 0.10)
Mineral mix[2]	1.0	1.3	12.0 (11.0, 15.0)
Selenium intake (mg/heifer/d):			
Actual	1.3	2.5	
Intensive dairy systems (NRC, 2001)	2.7	4.7	
Pastoral dairy systems (Grace, 1992)	0.5	0.7	

[1]Before calving, grass silage was fed to heifers in 3 herds, and grass hay in 1 herd.
[2]Selenium as sodium selenite.

Species presumptively identified as staphylococci were tested by the catalase test, tube coagulase method and deoxyribonuclease degradation test in DNase agar plates. *Staphylococcus aureus* was identified by positive catalase, coagulase reaction and DNase tests. CNS were identified by a negative coagulase reaction and a negative DNase tests. Catalase reaction and Christie, Atkins, Munch-Petersen test were performed for the classification of isolates presumptively identified as streptococci. When necessary, the SVA Strep was used to classify streptococci according to their biochemical properties (SVA Strep, SVA, Uppsala, Sweden). Gram-negative isolates were classified as *Escherichia coli* when negative to oxidase test and positive to the production of ß-D-glucuronidase and indole (PI Test, SVA, Uppsala, Sweden). Strains that were negative were further tested by the API20E identification system (bioMérieux SA, Marcy l'Etoile, France), which permits a rapid species identification of *Enterobacteriaceae* and other Gram-negative organisms (Oliver et al., 2004).

Milk SCC was determined using a Combifoss 5300 cell counter (Foss Electric, Hillerød, Denmark). Data were expressed as natural logarithm of SCC in thousands/mL (LnSCC) to approximate the normal distribution. LnSCC was backtransformed for presentation purposes.

Selenium in pasture and concentrate was evaluated at the beginning of the study by the hydride generation atomic absorption spectrometry method (Clinton, 1977), and expressed in milligrams/kg DM.

The Se status of heifers was evaluated through the activity of GPx by a kinetic method (Ransel, Randox Lab, Crumlin, UK). This selenoenzyme is a biomarker of Se status and its activity was measured using the method of Paglia and Valentine (Paglia and Valentine, 1967) with slight modifications (Ceballos et al., 1999). Briefly, hemoglobin in fresh blood was measured by the cyanomethemoglobin method prior to GPx analysis. The enzyme activity is proportional to the decrease in absorption at 340 nm after the oxidation of NADPH into NADP.

Results are expressed in U/g Hb, considering an activity below 100 U/g Hb to be indicative of a suboptimal Se status (Ceballos et al., 1999).

6.3.4   Definition of Intramammary Infection

A quarter was considered to have an IMI at calving when ≥ 1 cfu/0.05 mL (equivalent to ≥ 20 cfu/mL) of a pathogen was obtained from both foremilk samples collected at calving and one day after. An IMI in the 28-d period after calving was established when a pathogen was cultured, as described above, in two out of three consecutive samples (i.e. among 7, 14 and 21 DIM or among 14, 21 and 28 DIM after calving). A quarter with an IMI at calving was considered cured for a pathogen if the sample was free of the pathogen in question on, at least, two consecutive tests during the 28-d study period (Chapter 5). An IMI established after calving was considered as a new IMI if the quarter did not have an IMI at calving (for the pathogen in question) or if an IMI at calving was cured before the (new) IMI was established. Samples containing more than two bacterial species were considered as contaminated, and were not informative of IMI status.

6.3.5   Statistical Analysis

Glutathione Peroxidase. The repeated measurements of GPx (before treatment, 14 and 28 DIM) of each heifer were analyzed using linear mixed models (Dohoo et al., 2009). An unstructured correlation structure for the dependence within a series of observations for blood GPx activity within a cow was evaluated. The model also included fixed categorical effects of herd, treatment group, time (days), and the interaction of treatment and time. Because of significant interaction of treatment and time, effects of treatment were evaluated at each time point using F-tests, and effects of time were evaluated similarly within each treatment group.

Furthermore, model-based pairwise comparisons between all treatments by time combinations were carried out with a Bonferroni adjustment for multiple testing.

Incidence Risk of New Intramammary Infections and Clinical Mastitis. The prevalence of IMI at calving was calculated as the number of IMI divided by the number of quarters sampled at that specific time. A logistic regression model combined with generalized estimating equations (GEE) to account for within-heifer clustering (exchangeable correlation structure) was used to evaluate any differences between unsupplemented control heifers and Se-supplemented heifers, while including also fixed effects of herds. The coefficients of the regression model were expressed as population-averaged odds ratios (OR), which gives the effect of Se supplementation on having an IMI at calving across all heifers (Dohoo et al., 2009). As assuming that missing values were missing completely at random, they should not introduce any biases in the GEE estimates values (Dohoo et al., 2009).

The incidence risk of having a new IMI was calculated as the probability that an individual quarter had a new IMI in the 28-d period after calving, considering only quarters free of IMI with the pathogen of interest at calving. The quarter was the unit of interest, and clustering within heifer was accounted for by GEE estimation in a similar logistic regression model as above, and including also fixed effects of herds.

Incidence of CM at calving, and incidence of CM in the first month of lactation were analyzed by similar logistic regression models as described above, and herd fixed effects were also included. Comparisons of incidence of CM in the first month of lactation between unsupplemented and Se-supplemented heifers were based on likelihood-ratio following random effects logistic regression analyses, because one of the treated groups had no incident cases during that period. All analyses were carried out using the 'xtgee' and 'xtlogit' commands of Stata version 11.0 (Stata Corp, College Station, TX, USA).

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Somatic Cell Count. The proportion of quarter samples with SCC below different cut-offs (i.e. < 150,000; < 250,000; < 750,000; and < 1,000,000 cells/mL) was calculated for each experimental group and time point (Barkema et al., 1999; Piepers et al., 2010).

Geometric mean SCC of 593,000 cells/mL has been found in non-infected quarters at calving (Barkema et al., 1999), while later in lactation a cut-off point of approximately 200,000 to 250,000 cells/mL has been established as optimal to reduce diagnostic error in distinguishing between non-infected and infected quarters (Schukken et al., 2003). Therefore, the odds of having a quarter with SCC > 500,000 and > 250,000 cells/mL at calving and after calving, respectively was analyzed by a logistic regression model with fixed effects of supplementation, time and herd. Clustering within quarters and heifers was accounted for by the alternating logistic regression algorithm (Kleinbaum and Klein, 2002; Dohoo et al., 2009). Analyses were carried out using the GENMOD procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

The repeated measurements of SCC on each heifer were also analyzed using linear mixed models (Dohoo et al., 2009). A preliminary analysis of SCC revealed a large variation between data collected around the day of calving and in the 28-day study period. Consequently, two separate analyses were carried out for the analysis of SCC. One analysis was performed for those samples collected at calving and the day after, and the other one included the information on SCC at 7, 14, 21 and 28 DIM. The correlation structure consisted of two parts: unstructured correlations between quarters, and additional unstructured and first order autoregressive correlations across the first two time points and the time points after calving, respectively. Fixed effects of herd, treatment group, time, and their interactions were included. All models were evaluated by examining the standardized residuals, and Box-Cox transformations of the outcome were explored whenever the model assumptions were not fully met. The final analysis was carried out on natural log scale for SCC at calving, and on a power-transformed scale for SCC in

the 28-d study period; all estimates were converted to natural log SCC (LnSCC), and

backtransformed to SCC for presentation purposes. The linear mixed model analysis for GPx

and LnSCC were carried out using the MIXED procedure of SAS version 9.2 (SAS Institute Inc,

Cary, NC, USA).

6.4 Results

The mean interval from the beginning of the study to calving was 30 (95% CI: 25, 36),

and 33 (95% CI: 29, 36) days in the unsupplemented heifers, and Se-supplemented heifers,

respectively. Average milk yield at 28 DIM (end of the study period) was 23.4 ± 0.9 kg in

unsupplemented heifers, while Se-supplemented heifers produced 1.2 ± 0.9 kg more. Analysis

by contrasts in a linear model (including also herd effects) showed that the difference between

control and supplemented heifers was not significant ($P = 0.20$), nor was there a significant

difference between the two Se sources ($P = 0.78$).

Mean blood GPx activity was similar among groups at the beginning of treatment ($P =$

0.14). Selenium treatment, time after treatment, and their interaction had a significant effect on

blood GPx activity ($P = 0.02$). Mean blood GPx activity was significantly higher in heifers

treated with barium selenate than in unsupplemented controls and heifers fed Se yeast at 14

DIM, and significantly higher than controls at 28 DIM (Figure 6.1). Blood GPx activity also

increased significantly over time in all groups until the end of the study (Figure 6.1), and was

116 U/g Hb (95% CI: 65, 167 U/g Hb) higher in the heifers treated with barium selenate

compared to the mean activity in unsupplemented controls, and 74 U/g Hb (95% CI: 23, 125)

higher than the mean activity in the heifers fed Se-yeast at 28 DIM.
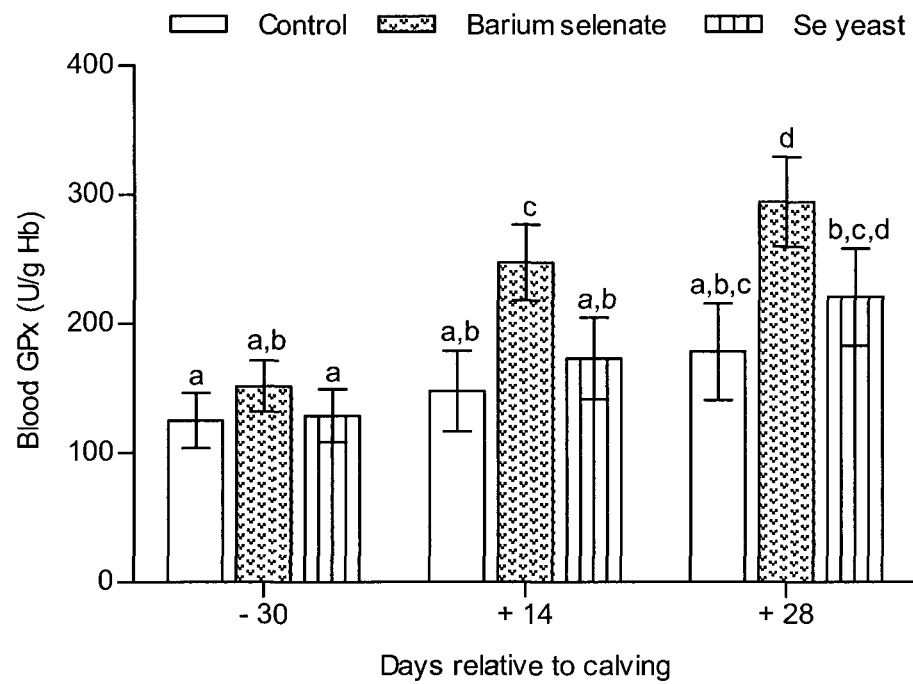
Figure 6.1. Least square means with SE of blood glutathione peroxidase (GPx, U/g Hb) activity in unsupplemented pastured heifers (n = 49) or supplemented with barium selenate (n = 46) and Se yeast (n = 45) one month before calving.
[a,b,c,d]Different superscripts differ significantly (adjusted $P < 0.05$).

6.4.1   Intramammary Infection and Clinical Mastitis

The effect of Se supplementation on IMI was evaluated on 1846 (55% of the original dataset) remaining samples, as 1053 were lost, and 461 did not match the criteria to establish an IMI.

A total of 780 quarter foremilk samples collected at calving were cultured. Bacterial growth occurred in 209 samples (27%), 81 samples were contaminated (10%), and a pathogen could not be classified in 8 samples (1%). CNS were the most frequently isolated group of bacteria (70%), followed by *E. coli* (9%) and *Streptococcus uberis* (6%). Prevalence of IMI at calving tended to be lower in Se-supplemented heifers compared to unsupplemented controls (OR: 0.5; 95% CI: 0.2, 1.2). No differences were observed among herds ($P = 0.82$).

The proportion of infected quarters at calving that cured was 69 and 74% for unsupplemented and Se-supplemented heifers, respectively. However, the odds of cure was not affected by treatment (OR: 1.5; 95% CI: 0.2, 13.2). Quarters infected with *Strep. uberis* and other pathogens at calving did not cure over the study period (Table 6.2), while CNS IMI at calving lasted 16 d on average, ranging from 14 d to 28 d.

A total of 1066 quarter milk samples collected during the 28-d study period after calving were cultured, and bacterial growth was found in 172 samples (16%). Seventy-four samples (7%) were contaminated, and a pathogen could not be classified in 21 samples (2%). Coagulase-negative staphylococci were the most common isolations (63%), while *Staph. aureus*, *Streptococcus dysgalactiae* and *Strep. uberis* were isolated in 16% of the samples. Twenty-six IMI were found in the 28-d study period, 19 of them new IMI. Even though new IMI were numerically higher in Se-supplemented heifers, supplementation did not significantly increase the overall odds of having a new IMI in the 28-d study period (OR: 2.2; 95% CI: 0.7, 7.4). No new IMI were found in one of the herds after calving; also, in the remaining herds, the odds of having a new IMI after calving differed among herds ($P = 0.02$). Because of the low number of

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new infections, the pathogen-specific effect of supplementation could only be assessed for CNS and did not show any statistical difference (OR: 2.5; 95% CI: 0.6, 11.4).

Eleven cases of CM were detected at calving of which four occurred in the unsupplemented control heifers, and seven cases in the Se-supplemented groups (OR: 1.1; 95% CI: 0.2, 6.4). During the first month of lactation, 13 new cases were detected, six of them occurred in the unsupplemented heifers, no new cases were found in the barium selenate treated group in the 28-d study period, and seven occurred in the Se yeast treated heifers (Table 6.2). The comparison of CM incidence among groups was close to significant (P = 0.06), and also comparisons between the barium selenate-treated group and each of the other groups failed to achieve statistical significance after a Bonferroni-adjustment for multiple comparisons.

6.4.2 Somatic Cell Count

A total of 998 quarter foremilk samples collected at calving and one day after, and 2108 quarter milk samples collected between 7 and 28 DIM were analyzed for SCC. The proportion of quarters with SCC below different cut-offs at calving and after calving in the treatment groups is presented in Table 6.3. Geometric mean SCC decreased from 682,000 at calving to 368,000 cells/mL one day after calving ($P < 0.001$), and then gradually decreased until the end of the 28-d follow-up period ($P < 0.001$; Figure 6.2). Selenium supplementation, regardless of Se source, tended to reduce SCC one day after calving ($P = 0.08$; Figure 6.2). However, there was no effect of supplementation on LnSCC during the 28-d period after calving ($P = 0.48$; Figure 6.2).

Table 6.2. Quarter-level prevalence of intramammary infection and incidence of clinical mastitis at calving, number of cured quarters, and new infections and clinical mastitis cases in the first month of lactation in pasture-based primiparous heifers supplemented with selenium before calving and unsupplemented control heifers.[1]

	Unsupplemented			Barium selenate			Se yeast		
	C^2	Cured[3]	L^4	C^2	Cured[3]	L^4	C^2	Cured[3]	L^4
Staphylococcus aureus	0	0	0	0	0	1	0	0	1
Streptococcus dysgalactiae	0	0	1	0	0	0	1	0	0
Streptococcus uberis	1	0	0	1	0	0	0	0	0
Escherichia coli	4	4	0	0	0	0	0	0	0
CNS	17	14	2	8	6	4	12	11	8
Other pathogens	4	0	0	0	0	1	1	0	1
Total	26	18	3	9	6	6	14	11	10
Clinical mastitis cases	4	-	6	4	-	0	3	-	7
Total quarters at risk[4]	126	-	102	83	-	83	118	-	113

[1]Only quarters with complete set of samples were included.
[2]Number of quarters with an IMI at calving.
[3]Number of cured quarters that had an IMI at calving.
[4]Number of quarters with new IMI in the first 28 DIM.

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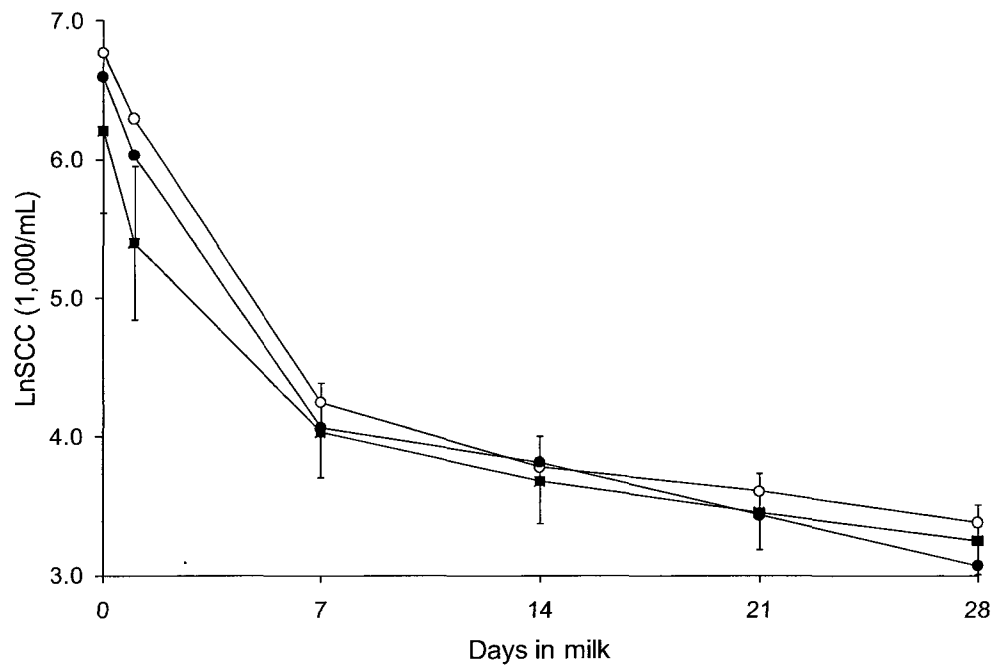
Figure 6.2. Model-based estimated somatic cell counts (LnSCC) in milk in pastured heifers, which were either unsupplemented ($\circ$, n = 49) or supplemented with barium selenate ($\blacksquare$, n = 46) or selenium yeast ($\bullet$, n = 45) one month before calving. For clarity, 95% confidence interval bars are shown only for the barium selenate group.

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Table 6.3. Percentage of quarters with somatic cell counts (SCC) below different cut-off points according to days in milk (DIM) in pasture-based primiparous heifers supplemented with selenium before calving and in unsupplemented controls.

DIM	SCC (1,000 cells/mL)											
	< 150			< 250			< 500			< 1,000		
	C[1]	BS[2]	Se Y[3]	C[1]	BS[2]	Se Y[3]	C[1]	BS[2]	Se Y[3]	C[1]	BS[2]	Se Y[3]
0	20	30	26	30	32	36	40	51	50	53	57	58
1	27	41	34	38	50	44	49	65	59	61	73	68
7	70	69	73	80	80	78	90	86	86	94	89	89
14	77	74	76	86	89	89	91	92	95	93	94	96
21	77	82	81	84	91	92	90	94	96	94	96	98
28	77	77	84	88	87	94	93	94	99	96	96	99

[1]C = Control group.
[2]BS = Barium selenate group.
[3]Se Y = Selenium yeast group.

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Table 6.4. Multivariable regression model of the effect of selenium (Se) supplementation, sampling time and herd on the prevalence of quarters with somatic cell counts (SCC) > 500,000 cells/mL at calving, and SCC > 250,000 cells/mL in the first 28 days in milk (DIM) in pasture-based primiparous heifers supplemented with selenium before calving and unsupplemented controls.

	OR[1]	95% CI[2]	*P*-value
SCC > 500,000 cells/mL at calving			
Treatment			0.04
Unsupplemented	1		
Se-supplemented	0.6	0.3, 1.0	
Herd			0.02
1	1.5	0.7, 3.0	
2	0.5	0.2, 0.9	
3	0.7	0.4, 1.3	
4	1		
Sampling (DIM)			< 0.01
0	1		
1	0.6	0.5, 0.8	
SCC > 250,000 cells/mL at 7-28 DIM			
Treatment			0.20
Unsupplemented	1		
Se-supplemented	0.7	0.5, 1.2	
Herd			< 0.01
1	0.5	0.2, 1.1	
2	1.6	1.0, 2.8	
3	2.5	1.3, 4.6	
4	1		
Sampling (DIM)			< 0.01
7	1		
14	0.5	0.3, 0.8	
21	0.5	0.3, 0.7	
28	0.4	0.3, 0.7	

[1]Odds ratio.
[2]95% confidence interval.

A significant reduction in the odds of having a quarter with SCC > 500,000 cells/mL at calving was found in Se-supplemented heifers compared to unsupplemented controls (Table 6.4). However, Se supplementation did not affect the odds of having a quarter with SCC > 250,000 cells/mL during the 28-d after calving. The odds of a quarter having a SCC below the selected cut-off points differed among herds at calving, and after calving (Table 6.4).

6.5   Discussion

Blood GPx activity increased over time in Se-supplemented heifers, and was higher in heifers supplemented with barium selenate. Selenium supplementation also tended to decrease the prevalence of IMI and average SCC at calving; however, no differences were found in the incidence risk of new IMI or SCC among groups in the 28-d follow-up period after calving.

A higher blood GPx activity was found in barium selenate-treated heifers from 45 d after treatment, concurring with other studies (Grace et al., 2001), suggesting that supplemental Se released from the site of injection was effectively incorporated into the erythrocytes. However, Se incorporation into GPx structure during erythropoiesis is a slow process (Grace et al., 2001). Thus, a lag (~ 45 d) in the change of blood GPx activity occurs following changes in dietary Se intake (Thompson et al., 1981; Grace et al., 2001). The first blood GPx analyses, averaging 135 U/g Hb, reflected an adequate dietary Se intake prior to the beginning of the study based on Chilean reference ranges (Ceballos et al., 1999), further analyses at 14 and 28 DIM reflected an increase in the basal intake due to supplementation of close-up diets with concentrates and mineral mixes.

No differences in blood GPx activity were found between heifers fed Se yeast and unsupplemented controls. Other studies have found that organic Se may be metabolized in a

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different way compared to inorganic sources, indicating that Se yeast is non-specifically incorporated into selenoproteins (Awadeh et al., 1998). Consequently, supplementing Se yeast does not necessarily mean a concurrent increase in blood GPx activity.

Prevalence of IMI tended to be lower at calving in Se-supplemented heifers when compared to unsupplemented controls. Selenium supplementation was highly protective at calving in first-lactation heifers in an intensively managed North American herd, reducing the prevalence of staphylococcal IMI by 42% (i.e. 91% of these were CNS, and isolated coagulase-positive staphylococci were *Staph. hyicus* or *Staph. intermedius*), while no differences in the prevalence of streptococci IMI and *E. coli* IMI were found (Smith et al., 1985). Additionally, in that study the incidence of CM was lower, IMI were of a shorter duration, and SCC was lower compared to unsupplemented heifers. Notwithstanding, heifers in the trial of Smith et al. (1985) were fed an equivalent Se intake of 2 µg Se/kg LW/d and a subcutaneous injection of 0.1 mg Se/kg LW, which may be higher compared to the current NRC (2001) recommendation. Achieving a Se intake close to NRC (2001) recommendation in dairy cattle from Southern Chile would be unlikely due to low Se concentrations in pastures and crops in that region; therefore, heifers in our study received a lower equivalent Se intake than reported in other studies (Smith et al., 1985; Malbe et al., 1995).

The slight effect of Se supplementation on IMI at calving, and on CM in the 28-d after calving, in particular the absence of incident cases of CM in heifers injected with barium selenate in this period, can be linked by several factors. Mineral supplementation, particularly with Se, enhances leukocyte function and the mammary immune response to mastitis pathogens (Hogan et al., 1990; Piepers et al., 2009). The effect on the udder immune response is mediated through the expression of selenoproteins with antioxidant properties (Sordillo et al., 2007). The depletion of critical antioxidant defenses, such as GPx, may predispose transition cows to mastitis during early lactation. Glutathione peroxidase reduces lipid hydroperoxides generated

during the respiratory burst, protecting leukocytes from killing themselves (Sordillo et al., 2007). In addition, Se supplementation enhances the production of chemoattractants, and polymorphonuclear cells (PMN) influx into the udder (Erskine et al., 1989), favors the production of inflammatory mediators (Cao et al., 1992), and enhances the bactericidal capacity of the neutrophils (Gyang et al., 1984; Erskine et al., 1989) which together explain a lower prevalence of infection at calving, less severe IMI, and a rapid elimination of IMI in Se-supplemented animals. The low incidence of CM in the first four weeks after calving in heifers injected with barium selenate might be the net result of differences in the incorporation of Se into selenoproteins with antioxidant properties. As mentioned, there are differences in the way different Se sources are metabolized (Awadeh et al., 1998), Se yeast is not incorporated directly into specific selenoproteins related to the immune response of the mammary gland.

The lack of effect of Se supplementation on the incidence of IMI can be partially explained by the power of the study; there was sufficient power to detect a 40% change in the risk of new IMI between groups, which is a relatively large effect. Second, Se status was considered adequate when the study started. However, blood GPx activity at that point was within the range at which we previously found a beneficial effect of Se supplementation on udder health (Kruze et al., 2007). A third reason could be that the basal Se intake, as indicated by an increase in blood GPx even in the unsupplemented controls, was not constant over the study period due to feeding concentrates and mineral mixes immediately before calving.

Pathogen-specific differences in the PMN influx to the udder in response to IMI may be another factor that explains the lack of a beneficial effect of Se on the odds of having a new IMI in the first month of lactation. A massive and rapid influx of PMN to the udder was found in Se-supplemented cows after *E. coli* IMI (Erskine et al., 1989). The influx of PMN was slower in *Staph. aureus* IMI, but PMN were more efficient at killing *Staph. aureus* in Se-supplemented cows than were PMN in unsupplemented controls (Erskine et al., 1990). In addition, early

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observational studies indicated that the inflammatory response to CNS IMI is less pronounced than the response to IMI caused by major pathogens (Barkema et al., 1999). In our study, however, the prevalence and incidence of *Staph. aureus* and CNS IMI were lower than reported in previous studies, and no new *E. coli* IMI were found.

The association between the udder immune response and nutrients other than Se could also explain our results. Even though plasma tocopherol values vary with the stage of lactation, dropping by 50% at the beginning of lactation, values started to increase after 20 DIM (Weiss et al., 1990). This profile is explained by changes in the DMI around calving. However, cows raised in pastoral systems will typically experience more than sufficient intake of vitamin E when fresh forage is fed (Weiss, 1998), and the higher vitamin E intake appears to have a sparing effect on Se requirement (Wichtel, 1998); however this relationship has not been exactly quantified (NRC, 2001). A low incidence of Se-responsive disorders under pastoral conditions, such as subclinical or clinical mastitis, may be the result of high concentrations of vitamin E in pasture (Wichtel, 1998). Peer-reviewed literature concerning the dynamics of IMI in unsupplemented and Se-unsupplemented cows under pastoral conditions comparable to those of our study is scarce. However, similar results were found in a trial conducted in the South of Chile enrolling primiparous and multiparous cows, in which Se supplementation with barium selenate before calving did not affect the risk or rate of new IMI during the subsequent lactation (Chapter 5).

Somatic cell count is elevated shortly after calving even in the absence of IMI, declining very rapidly in the first 6 milkings after calving (Barkema et al., 1999; Piepers et al., 2010), and reaching normal levels between two and four weeks of lactation (Dohoo, 1993). A relatively steep decline in mean LnSCC was found from calving up to seven DIM, and then a gradual reduction from seven DIM onwards, which concurs with previous studies (Barkema et al., 1999; Sampimon et al., 2009). The post-calving decrease in SCC is the net result of a combination of

the reduction in the number of false-positive elevated SCC and a decline in the prevalence of IMI (Dohoo, 1993). In our study, Se supplementation caused a reduction in the prevalence of IMI at calving, which might explain a lower SCC found in Se-supplemented heifers; these results support the conclusion from earlier studies that found a negative relationship between Se status and SCC in milk (Smith et al., 1985; Malbe et al., 1995).

Differences in SCC after the first week of lactation have an impact on lactational SCC, milk production, and culling rate (De Vliegher et al., 2004; De Vliegher et al., 2005a; De Vliegher et al., 2005b). Although SCC decreased shortly after calving in Se-supplemented heifers, geometric mean SCC was not affected by Se source. In addition, no differences were found between unsupplemented controls and Se-supplemented heifers in the 28-d follow-up period after calving. Again, the study design may partially explain these results. Although the sample size was large enough to detect differences in blood GPx activity and a difference of 40,000 cells/mL, the data did not reflect SCC differences of this magnitude between unsupplemented controls and Se supplemented heifers. In addition, results of North American and European studies appear to have been influenced by a limited number of herds where a reduction of disease incidence (i.e. primarily CM caused by environmental pathogenic bacteria) has been reported with very high levels of supplementation. Moreover, early studies in intensive systems started with a higher pre-treatment prevalence of IMI than that observed in our study, thus the pathogen challenge in this study might have been lower (either type or degree) than comparable studies of intensively raised heifers and cows.

6.6   Conclusions

Selenium supplementation one month before calving increased blood GPx activity, slightly reduced the prevalence of IMI at calving, and lowered SCC at the time of calving, regardless of Se source (e.g. injectable barium selenate or Se yeast). Selenium supplementation did not affect the odds of new IMI, new CM; nor did it influence SCC during the balance of the first month of lactation in pastured first-lactation heifers. Nevertheless, barium-selenate treated heifers had no incident cases of CM in the first month of lactation. Feeding diets with < 0.2 mg/kg DM Se (~ 60% of the current NRC recommendation) appears to be responsive to Se supplementation before calving, especially for improving udder health shortly after calving in first-lactation heifers grazing low-Se areas.

6.7   Acknowledgements

6.8   References

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7 **GENERAL DISCUSSION**

7.1 Introduction

The essentiality of Se for preventing degenerative diseases was established in mid 1950s as a result of studies conducted in rodents and chicks by Klaus Schwarz and Calvin M. Foltz (Schwarz et al., 1957; Schwarz and Foltz, 1957; Schwarz and Foltz, 1958). Practical problems associated with deficiency or marginal intakes of Se were recognized in cattle and sheep soon after (Muth et al., 1958; Wolf et al., 1963; Andrews et al., 1968). The role of Se in udder health was established in 1980s (Smith et al., 1984; Smith et al., 1985). Even though the beneficial effect of Se supplementation had been recognized since 1950s, it was not legal to supplement cattle rations in the United States until 25 years later (Ullrey, 1980). In 1979, an amendment to previous regulations of the Food and Drug Administration included the following (Ullrey, 1980):

"... Selenium (as sodium selenite or selenate) may be added to feed as follows:
...
(5) Dairy cattle: (a) In complete feed at a level not to exceed 0.1 ppm ..."

Over the last 30 years, higher rates of Se supplementation have been employed, likely because producers and advisers have become increasingly more knowledgeable of dairy nutrition, and more aware of the importance of Se supplementation specifically, and a greater range of supplementation products have been aggressively marketed. Today we may be working with a dairy cattle population where the ranges of Se status are substantially different than in the experimental herds when a negative relationship between Se status and Se-responsive disorders was first established (Schwarz et al., 1957; Oldfield et al., 1960; Smith et al., 1984). Nonetheless, there continues to be a degree of uncertainty regarding what constitutes physiologically and economically *optimal* Se nutrition in dairy cattle. At the same time, our understanding of the diverse roles for Se in mammalian metabolism has greatly expanded the possible mechanisms through which Se might influence dairy cattle production and health. Thus

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the main objective of this thesis was to contribute to a better understanding of the association of Se status and Se supplementation with milk Se concentration and measures of udder health in dairy cattle under different dairy management systems.

Interest in milk Se concentration and udder health arose for three reasons: first, the wider use of organic forms of Se supplementation in the past 10 years has confirmed that transfer of dietary Se to milk can be readily enhanced. Secondly, Se appears to be important in the regulation of many aspects of the immune response, and there is a renewed interest in the effects of Se in blood, milk and tissues on udder defense, and how these concentrations might best be manipulated at the farm level. Finally, there is an increasing interest, world-wide, in developing methods for production of functional foods for human consumption, which are defined as foods that encompass potentially healthful products for humans including any modified food (e.g. Se-enriched milk) or ingredient that may provide a health benefit beyond the traditional nutrients.

The work presented in this thesis addressed these topics through five studies. First, a systematic review and a meta-analysis were developed to summarize the evidence for the effect of oral Se supplementation on milk Se concentration (Chapter 2). The association of Se status with udder health was examined in Atlantic Canadian dairy herds (Chapter 3). This work was extended by examining this relationship at the herd level in samples of herds from all major dairy regions of Canada (Chapter 4).

In contrast to the above studies which related primarily to intensive North American dairy management systems, two clinical trials were conducted in exclusively pastured herds to evaluate whether the typical response in blood GPx activity observed after Se supplementation in pastured dairy cattle is associated with improved udder health. In both trials, depot injectable barium selenate was chosen as an experimental treatment because long-acting forms of supplementation are highly relevant to herds in pasture-based systems, where supplementation

through addition of Se to daily rations is not always practical. In the first of these trials, pre-calving supplementation with barium selenate was compared to no supplementation in cows, and measures of udder health in these cows were examined during the subsequent lactation (Chapter 5). In the second trial, injectable barium selenate and oral organic Se were compared to supplementation with a more traditional source (i.e. oral sodium selenite) in primiparous cows, and measures of udder health of these heifers were followed during the periparturient and early lactation periods (Chapter 6).

7.2   Milk Selenium Concentration

The meta-analysis of the effect of oral Se supplementation on milk Se concentration supported the contention that individual cow milk Se concentration increases after oral Se supplementation (Chapter 2). Milk Se concentration was higher when organic forms (i.e. Se yeast) were fed compared to traditional inorganic sources (e.g. sodium selenite/selenate). Therefore, Se supplementation not only improves the Se status of the cow, but also can assist in meeting the dietary Se requirement of consumers of dairy products.

The interest in the role of Se in human nutrition has increased in the last three decades, as a lower Se intake has been associated with Keshan disease and Kashin-Beck disease, and some juvenile cardiomyopathies that occur in certain low Se regions (WHO/FAO, 2004). The recommended daily Se intake for humans to prevent Se-responsive diseases is, depending upon gender and age, between 26 and 55 μg for adults (Institute of Medicine, 2000; WHO/FAO, 2004). Even though milk contributes to the Se intake of humans, concentrations are lower in the milk of cows from regions that have low soil Se concentrations, and milk from these regions cannot contribute materially to meeting daily requirements (Sánz-Alaejos and Díaz-Romero,

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1995). The meta-regression analysis of milk Se concentration on continent, source and dose of Se (Chapter 2), indicated that if cows in North America are fed approximately 11 mg/cow/d of Se (as sodium selenite/selenate) or 6 mg/cow/d of Se (as Se yeast) this will provide at least 10% of the minimum recommended human dietary Se intake in a 100 mL serving of whole milk. Therefore, an increased milk Se concentration makes milk an effective delivery vehicle for health-promoting specific nutrients, such as Se, and thus dairy products become more attractive and valuable to dairy consumers (Knowles et al., 2006).

While the effect of Se supplementation on milk Se concentration has been established for some time, the relationship between oral Se intake and milk concentration, and the factors that might influence this relationship had not been previously characterized. Milk Se concentrations have successfully been used to assess Se status of cattle (Binnerts et al., 1984; Grace et al., 2001; Wichtel et al., 2004). However, determination of Se concentration in milk is not widespread for several reasons. Concentrations are at the low end of the detection limit for several analytic methods, milk is a difficult sample to work with because of the fat and protein content, and it is not homogenous over time. However, milk Se analysis has the benefit that milk is easily collected, in many cases already collected for other purposes, is highly responsive to changes in Se intake (Chapter 2), and bulk tank milk Se concentration (BTSe) is a representative measure of Se intake of lactating cows.

A number of factors should be considered in the interpretation of milk Se concentration when using it to help establish the herd- or cow-level Se status, as milk Se concentration was highly variable among studies (Chapter 2). The continent where the study was carried out, source, and dose of Se were significant contributors to the between-study variance (Figure 2.3). Stage of lactation and milk yield may be significant contributors to the variation in milk Se concentration, as milk Se concentration may appear low at the beginning of lactation and in high-producing cows (Wichtel et al., 2004). Although the results of the meta-analysis indicated

that stage of lactation explained 43% of the between-study variance, this variable was not significantly associated with milk Se concentration (Chapter 2). A dilution effect due to high milk yield seems to be also a plausible explanation for changes in milk Se concentration, but in the observational study about milk Se concentration in cows from Atlantic Canada the difference in milk yield was much lower than observed differences in milk Se concentration (Chapter 3). On the other hand, a higher DMI at the beginning of lactation or a higher rate of passage might affect Se absorption, resulting in lower milk Se concentration in early lactation.

7.3   The State of Selenium Nutrition in Canadian Dairy Cattle

In general, based on individual milk Se and BTSe concentrations in the actual population of selected individuals in Atlantic Canadian herds (Chapter 3, and in selected dairy herds across Canada (Chapter 4), appear to be adequate. Cow- and herd- level milk Se concentrations were higher compared to previous results (Fisher et al., 1980; Wichtel et al., 2004). However, this inference is not applicable to cow and herd populations beyond our target population because cows and herds were purposively selected (Chapters 3 and 4). Therefore, there is an undetermined uncertainty about how well the attributes of our study population reflect the attributes of a larger population of diary cows in Atlantic Canadian herds or the attributes of Canadian herds.

The current Se status may be the result of the widespread adoption of improved Se supplementation practices in the last decade, and the approval of organic Se for dairy cattle expanded the options for Se supplementation. In keeping with the finding of an adequate Se status in these herds, all dairy farmers included in the study in Atlantic Canada reported that they supplied Se in bred heifer, dry cow and lactating cow rations, with inclusion in concentrates being the preferred method of supplementation (Chapter 3). Previous authors found that the odds

of being a Se-adequate herd, as established by BTSe concentration, increases four times when Se is fed in commercial rations (Wichtel et al., 2004).

Seasonal variation was observed in herd- and cow-level milk Se concentrations (Chapters 3 and 4). Lower milk Se concentration (i.e. < 0.28 μmol/L) was found cattle with access to pasture in the summer months. Seasonal variation in the content of Se in forages and crops could lead in part to the seasonal differences found in herd- and cow-level milk Se concentrations. Low Se levels in pasture and crops in the grazing season can cause a sub-optimal Se intake leading to decreased milk Se concentration in cows with access to pasture. Variation in the amount of concentrate fed to dairy cows associated with turn out to pasture in the spring can lead also to low milk Se concentration (Ropstad et al., 1987; Miller et al., 1995; Wichtel et al., 2004). This means that time of the year and whether or not cows have access to pasture should be considered when assessing the risk of Se inadequacy for dairy herds.

7.4 Effect of Selenium on Udder Health

Selenium adequacy, as evaluated by individual milk Se concentration, was not associated with the overall odds of having a new intramammary infection (IMI) in the dry period (Chapter 3). Specifically, neither the incidence of *Staphylococcus aureus* nor CNS IMI in the dry period was affected by milk Se concentration at drying-off. However, the odds of new IMI caused by *Streptococcus* spp. and other Gram-positive pathogens increased with higher milk Se concentration, suggesting a pathogen-specific positive relationship between Se status in the dry period. Although not all literature provides support for a role of Se in mastitis, this is the first time a positive relationship between Se status and the odds of having a new IMI caused by *Streptococcus* spp. and other Gram-positive pathogens was found.

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Early studies supported the concept that the effects of Se supplementation on udder responsiveness to IMI may be pathogen-specific (Erskine et al., 1989; Erskine et al., 1990). Supplementation enhanced the influx of neutrophil into the udder and their phagocytic efficiency, resulting in lower bacterial numbers after an *Escherichia coli* IMI (Erskine et al., 1989). After a challenge with *Staph. aureus* the influx of polymorphonuclear cells (PMN) into the udder was lower when compared to an *E. coli* challenge, but bacterial growth rate was slower in supplemented cows, suggesting a more efficient killing ability of PMN in Se-supplemented cows (Erskine et al., 1990). However, Se supplementation did not affect the odds of having IMI caused by non-*agalactiae* streptococci (Smith et al., 1984; Smith et al., 1985).

The previous results support the idea that there are important variations in PMN response to infections caused by different pathogens (e.g. *E. coli*, streptococci, and *Staph. aureus*), which might partly explain the differences in pathogen-specific odds found in this thesis. An explanation for the effect of a higher risk of new IMI caused by *Streptococcus* spp. and other Gram-positive pathogens in the dry period in those cows with higher milk Se concentration remains unclear, and may be spurious. However, this relationship might be the result of a different pathogen challenge (either type or degree) compared to the pathogen challenge observed in previous studies where a beneficial role of Se on udder health was first observed. In addition, farmers with herds that have udder health problems (i.e. high bulk tank somatic cell count (SCC)) may intentionally supplement Se at a higher rate, or using organic forms (e.g. Se yeast) resulting in a non-causal relationship (i.e. a higher milk Se concentration associated with, but not causing, a higher odds of new *Streptococcus* spp. and other Gram-positive pathogens IMI in the dry period).

The excretory function of the mammary gland may explain the lack of effect of milk Se concentration on the overall odds of new IMI, or *Staph. aureus* and CNS new IMI in the dry period (Chapter 3). The mammary gland is not only a secretory organ but it can also have some

excretory capacity; approximately 15% of the daily Se intake is excreted in milk (Maus et al., 1980). Therefore, reaching a certain level of milk Se concentration does not guarantee an effective incorporation of Se into selenoproteins with a role against mammary pathogens. This observation concurs with a recent trial indicating an increased milk Se concentration after Se supplementation, but differences did not translate into an enhanced effect on neutrophil function or into the clinical response following an intramammary challenge with lipopolysaccharides (Weiss and Hogan, 2005).

Contrasting the results found at the cow-level, the probability of being a *Staph. aureus*-positive herd was significantly lower in those herds with high BTSe concentration (Chapter 4). The apparent beneficial effect of mineral supplementation, especially Se, on the udder health has been described elsewhere (Smith et al., 1984; Smith et al., 1985; Barkema et al., 1998; Piepers et al., 2009). An improvement of the killing ability of PMN in Se-adequate animals may explain these findings. Selenium supplementation resulted in a higher viability of neutrophils, a reduced catabolism of hydrogen peroxide, and greater protection against free radicals released during the bactericidal process, which together improve the ability of neutrophils to killing pathogens, such as *Staph. aureus* (Grasso et al., 1990; Sordillo et al., 2007; Sordillo and Aitken, 2009). Moreover, European studies have found that the whey of Se-supplemented cows has an antimicrobial activity against *Staph. aureus* (Ali-Vehmas et al., 1997; Malbe et al., 2006).

The relationship between Se status and lower odds of *Staph. aureus* IMI was not observed at the cow-level study (Chapter 3) or in selected Atlantic Canadian herds. This may be partially due to the use of BTSe as indicator of Se status, as BTSe is an aggregate (averaging) of Se data from lactating cows in the herd, not including data from other groups of cows that may be at risk of *Staph. aureus* IMI (e.g. cows close to drying off or dry cows).

Milk Se concentration reflected an adequate Se status in selected cows from Atlantic Canada, but marginal values can be observed when cows are turned out to pasture in the

summer. The Se status does not seem to be a determinant of udder health in cows from selected Atlantic Canadian dairy herds. Nevertheless, it is necessary to maintain Se adequacy (i.e. current levels of Se supplementation) to decrease the risk of being a *Staph. aureus*-positive herd.

7.4.1   Selenium Supplementation and Udder Health in Pastoral Systems

Selenium supplementation with a single injection of barium selenate before calving did not affect either the risk or incidence rate of having a new IMI in the subsequent lactation in multiparous pasture-based cows (Chapter 5). These results concur with previous observations in pastured herds (Grace et al., 1997; Wichtel et al., 2004; Kommisrud et al., 2005). Other authors have also reported no effect of Se supplementation on the response of health indicators of the mammary gland (Coe et al., 1993; Silvestre et al., 2007). Nutritional factors other than dietary Se intake, and non-nutritional factors can explain the lack of effect of Se on udder health, which is discussed below (Sections 7.4.3 to 7.4.6).

On the other hand, primiparous cows managed under pastoral dairy systems and supplemented with Se before calving, had a tendency toward a lower prevalence of IMI immediately after calving, regardless of Se source. However, Se supplementation caused no effect on the incidence of new IMI in the first month of lactation (Chapter 6).

A non-nutritional factor that might explain the lack of a significant effect of Se supplementation on the overall prevalence and incidence of IMI in Chapters 5 and 6 is the study design. Both studies were designed to have sufficient power to detect a difference of 40,000 cells/mL in SCC, and a reduction of 40% in the new IMI incidence based on previous results (Smith et al., 1984; Smith et al., 1985; Malbe et al., 1995). However, these are relatively large responses to obtain, likely highly variable between herds, and Se status may explain only a small part of the variation of these measures. The studies designed to estimate the effect of Se supplementation on udder health (Chapters 5 and 6), due to either a low sample size, or to loss of

samples after the fire in the microbiology laboratory of the Faculty of Sciences at Universidad Austral de Chile, might have had insufficient statistical power to detect an economically meaningful effect of Se supplementation, if one existed. The lack of statistical power in small trials has been described elsewhere, and it is not an exclusive problem of trials in animal science (Freiman et al., 1978; Pocock, 1982; Altman and Bland, 1995; Sterne and Davey Smith, 2001).

Trials evaluating the effect of Se supplementation on udder health reporting a beneficial effect have been variable in design (e.g. studies conducted in a limited number of herds, Se supplemented at variable rates, failure to account for clustering and potential confounders, etc) when compared to the design of the studies described herein (Chapters 5 and 6). Thus, the discrepancies between those results and results from previous studies where a beneficial effect of Se has been reported may arise from differences related to the study design. Future investigations should consider additional adjustments of the sample size to account for clustering within herd and cow, evaluating interactions, and potential confounders (Dohoo et al., 2009).

Studies reporting a "negative" effect (i.e. non-significant results) of any particular intervention are met with skepticism, even when the results come from studies with sound methodologies and have been subjected to an appropriate statistical analysis (Sterne and Davey Smith, 2001). Moreover, decisions regarding the efficacy of an intervention resides on an arbitrary division as to the significance or non-significance of results according to the commonly used cut-off point $P = 0.05$ (Sterne and Davey Smith, 2001). Thus the term "negative" wrongly implies that the study has shown that there is no difference, whereas all that has been shown is an absence of evidence of a difference (Altman and Bland, 1995), which is an indication that the data did not support reasonable rejection of the null hypothesis (Singh et al., 2008). Therefore, it has been suggested to use the confidence interval (CI) to quantify the association between any two variables A and B rather than a P-value when the results of the study are "negative", as CI convey information regarding direction, magnitude, and accuracy of effects. If the 95% CI

contains effects that are clinically trivial, the intervention does not provide meaningful results, but if the interval is wide enough to contain the null value and clinically important values, the study is inconclusive, and reflects an inadequate sample size (Altman and Bland, 1995; Sterne and Davey Smith, 2001; Singh et al., 2008). Additionally, the calculation of the CI, in particular the upper limit, has also been suggested as a tool to replace the post-study power calculations, as those can be misleading (Smith and Bates, 1992).

Another concern in the design of the studies described in Chapters 5 and 6 may be related to the fact that unsupplemented controls and Se-supplemented cows were pastured and milked together; if one of the groups has an increased incidence of IMI, it may influence the IMI risk in the other group. However, if a difference in udder health had been found, and the cows had not have been pastured and milked together, this difference could very well have been the result of other factors that were different between the two groups of cows.

Lastly, interpretation of blood GPx activity for cattle in Southern Chile has been based on previous publications (Ceballos et al., 1998; Ceballos et al., 1999). The effect of Se supplementation on udder health was evaluated in cows considered to have a low Se status, based on a blood GPx activity (i.e. < 100 U/g Hb). Values that fall around the low limit (i.e. marginally deficient range) may indicate a higher risk of Se-responsive disorders, but this does not guarantee a clinical or an economical response to Se supplementation (Wichtel et al., 1998b). An increase in blood GPx activity was found after Se supplementation; however, a higher blood GPx activity did not translate into a significant clinical response in the subsequent lactation (Chapters 5 and 6). This suggests that Se status does not seem to be a determinant of udder health over the typical range of Se intakes (i.e. ~ 0.2 mg Se/kg DM corresponding to 60% of the current NRC recommendation for dairy cows in intensive systems) of dairy herds from Southern Chile. However, Se supplementation, regardless of Se source, may be required in the close-up ration for first-lactation heifers to improve the udder health shortly after calving.

7.4.2 Selenium Status and Somatic Cell Count

Cow-level milk Se concentration was positively associated with mean SCC (Chapter 3), indicating that cows with higher milk Se concentration had higher SCC. Producers are more and more knowledgeable about the role of nutrition in optimizing udder health. Herds with a higher average SCC might intentionally supplement Se at a higher rate, or may use more organic Se, resulting in a non-causal association of higher concentration of Se in milk with higher SCC. However, that positive relationship between milk Se and SCC might also be the result of a more robust PMN influx into infected quarters in well-supplemented cows in Atlantic Canadian herds, but not necessarily resulting in a higher killing ability of those PMN. Other studies, however, are required to test this hypothesis. In contrast, results of field surveys and trials evaluating the effect of Se supplementation on udder health have demonstrated a negative association (Smith et al., 1985; Erskine et al., 1987; Malbe et al., 1995).

Although individual animal milk Se concentration was positively associated with SCC, no association was found between BTSe and bulk tank SCC (BTSCC) (Chapter 4). These results concur with previous studies that have found no association between Se status or Se supplementation and SCC in milk (Whelan et al., 1992; Coe et al., 1993; Wichtel et al., 1994; Grace et al., 1997; Bourne et al., 2008; Nyman et al., 2008). Nevertheless, a significant negative association between SCC and blood GPx activity had been previously reported in earlier studies from the United States (Erskine et al., 1987; Weiss et al., 1990).

In pasture-based dairy cows, a progressive increase in SCC was observed throughout lactation, but geometric mean SCC was not affected by a single injection of barium selenate before calving (Chapter 5). In grazing heifers, Se supplementation before calving tended to reduce SCC in milk one day after calving, but had no effect on SCC during the balance of the first month of lactation (Chapter 6).

The observed lack of relationship between supplementation and SCC response concurs with other studies (Ropstad et al., 1987; Whelan et al., 1992; Wichtel et al., 1994; Wichtel et al., 2004; Kommisrud et al., 2005). Studies in New Zealand have been performed in a dairy population characterized by a relatively low incidence of mastitis and other Se-responsive disorders in the face of a relatively low Se intake. Therefore, New Zealand researchers have suggested that the immune response is not greatly impaired in individual cows that are moderately deficient in Se (Wichtel, 1998a), or that Se requirement for maintaining an adequate udder health in heifers and cows raised in pastoral dairy systems might be less than that for animals in intensive dairy systems. Further studies are required, however, to confirm this hypothesis.

As our understanding of the immune response, antioxidant function, and the roles of Se improve, it becomes clear that Se is only one of many factors influencing the relationship between the immune response of the mammary gland and the damage caused by reactive oxygen species (ROS) (Miller et al., 1993; Sordillo and Aitken, 2009). To define a Se *requirement* becomes more and more difficult issue, as Se requirement may be highly variable under different management conditions (i.e. pastoral and intensive dairy systems), levels of production, and dependant on many factors, both measureable and unknown.

In particular, other nutritional factors than dietary Se intake, especially in pastoral dairy systems, may in part explain the discrepancies between the results presented herein and those coming from earlier studies, in particular studies from the United States. The most important factors are the following:

- Selenium status at the time of the study or before supplementation;
- Supply of other antioxidants, such as vitamin E;
- Dietary consumption of oxidants, such as polyunsaturated fatty acids (PUFA); or
- Differences in pathogen challenge between dairy systems.

These factors will be examined in more detail below.

7.4.3   Selenium Status Before Supplementation

A high proportion of cows (~ 80%) in Atlantic Canadian herds were considered to have an adequate Se status, as measured by the individual milk Se concentration (Chapter 3). However, a high milk Se content does not necessarily correlate with the function of Se in the mammary gland as a key component of the selenoenzymes related to the udder immune response. So, just because Se levels reached a certain level in milk, this does not mean that the selenoprotein activity in various mammary gland cell populations, either in the tissues or in the milk as SCC, has changed proportionately. Therefore, to conclude that Se status was a major factor associated with the overall immune response of the udder is not possible, if only milk Se concentration is measured. These results also suggest that in the participating herds, management and environmental factors other than Se status may determine udder health.

The finding of an increased SCC in milk associated with high milk Se concentration was not unexpected. A positive association between SCC in milk and an adequate Se status following an intramammary challenge with lipopolysaccharides has been recently described in North American cows (Weiss and Hogan, 2005). The migration of PMN into the udder following an infection is improved in cows with an adequate Se status (Smith et al., 1997). The peripheral PMN have a high activity of GPx, and contain a greater proportion of total blood Se (~ 73%) compared to the concentration in plasma (Scholz and Hutchinson, 1979). Therefore, the influx of blood leukocytes into the udder (e.g. following an IMI) may have caused a higher milk Se concentration.

The pastured cows forming the experimental population in the study described in Chapter 5 were considered deficient in dietary Se intake, as indicated by the content of Se in forages (< 0.05 mg/kg DM), and the mean herd-level blood GPx activity (< 100 U/g Hb). Even

though Se status of these cows was low, and blood GPx activity increased after Se-supplementation, there was no change in the udder health following supplementation. Nevertheless, at the beginning of our trial, the mean blood GPx activity was within the range of activity in which Se supplementation caused a beneficial effect on udder health in Chilean cows (Kruze et al., 2007).

Based on the estimation of daily Se intake and the whole blood GPx activity, the pastured heifers of Chapter 6 were considered to have an adequate Se status before the beginning of supplementation. Basal Se intake in heifers from Chapter 6 was higher than in cows from Chapter 5, which increased from 1.6 to 2.7 mg/heifer/d after calving. The basal dietary Se intake and the Se contributed by supplementation were enough to reach an optimal blood GPx activity, but a high blood GPx activity does not necessarily mean that the amount of Se required by other target cells or tissues to increase the activity of other selenoproteins involved in the immune response of the udder was reached. This supports the contention that maximal blood GPx activity may not be the best guide for assessing the adequacy of Se status for an optimal immune function. The evaluation of the blood Se level, and selenoprotein activity in white blood cells and mammary cells would help to determine the overall effect of Se status on known protective mechanisms (Sordillo, 2009. Personal communication)[6].

Similarly, high blood GPx activity does not invariably translate into adequate protection against oxidative stress. Studies conducted with non-ruminants have found that in severe cases of Se deficiency, where tissue levels of GPx fell to less than 1% of control, there was no evidence of tissue damage caused by ROS, which makes it difficult to propose a simple hypothesis for the activity of this enzyme, and an oxidative mechanism for pathogenesis (Arthur, 2000). If changes in tissue GPx are not necessarily associated with oxidative damage in Se-deficient animals, other mechanisms may be involved in the protection against oxidative stress.

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[6] Sordillo, L. 2009. College of Veterinary Medicine, Michigan State University. East Lansing, MI.

In addition, the form of Se in the selenoproteins is selenocysteine, and the dynamics of its incorporation into active selenoproteins will vary with respect to organ, and even cell types within an organ. Consequently, a higher availability of Se does not accurately predict its specific incorporation into the GPx or any other selenoproteins involved in the immune response (Sordillo, 2009. Personal communication)[6].

In summary, an adequate Se status was observed in purposively-selected Canadian dairy herds, likely to be the result of changes in the management practices associated with Se supplementation (Chapters 3 and 4). An increase in blood GPx activity was found after Se supplementation (Chapters 5 and 6), which is only an indication that Se intake was within an acceptable intake range. However, these results do not necessarily mean that Se-related biological functions were biologically or economically optimal. Thus, other analyses (e.g. selenoprotein, other than GPx, activity in white blood cells and mammary cells) may be required to address the overall effect of Se status on known immune protective mechanisms, and on udder health as well.

The analysis of Se status based only on the individual or herd milk Se concentration or on blood GPx activity, while convenient and backed by substantial experimental data, may not provide sufficient information to make conclusions about the association between Se status and the immune function of the udder under various management situations.

7.4.4   Selenium and Other Antioxidants Such as Vitamin E

Selenium and vitamin E are essential nutrients that share common biological activities in the protection of cells against ROS (Miller et al., 1993). The natural forage diets for cattle raised under pastoral systems are high in vitamin E, but values fall during forage conservation, as prolonged exposure to oxygen and sunlight increases the loss of vitamin E activity (Thafvelin and Oksanen, 1966). Vitamin E is also abundant in whole cereal grains, and by-products

containing the germ of the grain (Underwood and Suttle, 2001). Green and fresh forages, in particular alfalfa (*Medicago sativa*), are very good sources of vitamin E, carotenoids and other natural antioxidants for cattle. However, vitamin E content in forage is affected by stage of maturity at cutting and the time from cutting to dehydration (McDowell et al., 1996). The typical diets of cows in confined herds are often dependent upon ensiled forages as a source of roughage. Typically, these roughages and hays may contain 20 to 80% less vitamin E than freshly cut forages in the vegetative state (Kivmae and Carpena, 1973; NRC, 2001).

The results of early studies to evaluate the effect of Se and vitamin E supplementation on udder health suggested that vitamin E has sparing effects on the Se requirement relative to the intracellular killing of bacteria (Hogan et al., 1990). Hogan et al. (1990) suggested that the protection provided by vitamin E at the cellular membrane might spare the requirement for GPx by preventing the entry of ROS to the cytosol. Further, the percentage of quarters with new IMI at calving was lower in cows fed > 1,000 IU vitamin E/d and 0.15 mg Se/kg DM, compared to cows receiving low and intermediate concentrations of vitamin E (Weiss et al., 1997). However, that sparing effect has not been quantified to date (NRC, 2001).

The relatively low incidence of Se-responsive disorders, including mastitis, observed in New Zealand, despite the widespread Se deficiency of pastures, may be attributed to the typically high concentrations of vitamin E in pasture (Wichtel, 1996). Thus, the lack of clear evidence for the effect of Se supplementation before calving on the overall risk of new IMI and increased SCC in the subsequent lactation of pastured cows (Chapters 5 and 6) may in part be explained by the dietary intake of vitamin E, carotenoids and other antioxidants in fresh pasture. Although comparisons among countries are difficult to make due to the diversity in management systems for dairy farming, the typical systems of dairy production in New Zealand farms are probably most comparable to those dairy systems used in Southern Chile (Chapters 5 and 6) , so

it is not surprising that clinical response trials in these two countries have yielded quite similar results with respect to measures of udder health.

7.4.5   Dietary Oxidants (Polyunsaturated Fatty Acids)

The release of PUFA from cellular membranes, or a high intake of exogenous PUFA, have been associated with a high rate of ROS production, which provides an oxidant challenge, increasing the requirement for antioxidants, such as Se and vitamin E (Rice and Kennedy, 1988; Underwood and Suttle, 2001; Sordillo and Aitken, 2009). Uncontrolled production of ROS results in oxidative stress to tissues, and this model forms the basis for several Se-responsive disorders, including the impaired immune response of the udder (Miller et al., 1993; Sordillo and Aitken, 2009).

In mid 1970s, it was hypothesized that the intake of lush spring pasture containing a high concentration of PUFA may cause alterations in the lipid structure of cellular membranes, leading to myopathy within one week of turnout (McMurray and McEldowney, 1977). However, exogenous PUFA are not the only source of oxidants in the animal. For example, physiological stress in high-producing dairy cows due to a higher energy demand and an increased oxygen requirement makes high-producing dairy cows more prone to oxidative stress (Lohrke et al., 2005). The situation can be exacerbated in certain physiological conditions, such as the periparturient period (Miller et al., 1993; Castillo et al., 2005). Increasing the intake of dietary lipids, such as supplemental fat and oilseeds, is common practice in high-producing herds, and can also be a significant contributor to the load of ROS (Miller et al., 1993; Andrews et al., 2006). Models have been used to reproduce these effects: confined calves deficient in Se and vitamin E developed signs of myopathy after they were turned out on to fresh spring grass, and had 10-fold higher total plasma fatty acids than calves that remained indoors (Arthur, 1988).

However, an increase in PUFA is not the sole requisite for the development of signs of Se-responsive disorders (Arthur, 1988).

An increase in PUFA intake from pasture could be implicated in the studies of pastured dairy cows (Chapters 5 and 6), and in intensively managed cows when turned out on to pasture in the spring after the winter season (Chapter 3), or when ROS production increases in high-producing dairy cows due to a higher metabolic rate, and/or when fatty acids are used as energy supplements in intensive dairy systems (Chapters 3 and 4). The tendency toward improved udder health in heifers in the study in Chapter 6, but in no other studies, might be explained by some of the above factors, with the effects more obvious in growing heifers which may have a greater overall requirement for certain antioxidants, such as Se. Arguing against this hypothesis, forages with increased peroxidisable PUFA tend also to have increased protective concentrations of vitamin E (Hakkarainen and Pehrson, 1987; Wichtel et al., 1996) suggesting that dairy cattle fed a typical pasture diet would tend not to be at high risk for diseases associated with a high intake of PUFA, even in situations of moderate Se deficiency (Wichtel et al., 1996). Nonetheless, Wichtel et al. (1996) suggested that sudden reintroduction of cattle to immature lush pasture in the spring or fall, after prolonged periods of consumption of mature or conserved forages low in Se and vitamin E, might place cows at risk for Se-responsive disorders.

7.4.6   Pathogen Challenge Between Dairy Systems

Other causes for the lack of effect that are not necessarily related to the study design or nutritional factors may be the differences in the pathogen challenge between intensive and pastoral dairy systems (Sampimon et al., 2009). Either the type or degree of the pathogen challenge might have been insufficient compared to the challenges in other studies. For example, early studies on the effect of Se supplementation on udder health started with a higher

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prevalence of IMI at calving as compared with the prevalence found in the observational study in Atlantic Canadian herds (Chapter 3), and in the trial conducted in pastoral dairy systems in Southern Chile (Chapters 5 and 6). Additionally, previous studies found a higher incidence of environmental mastitis (Smith et al., 1984; Smith et al., 1985; Weiss et al., 1990), and heifers in the study of Smith et al. (1985) received Se at a higher rate than the current NRC (2001) recommendation. The number of new IMI caused by environmental pathogens, in particular *E. coli*, was low in quarters from cows in Atlantic Canada (Table 3.4), and no incident *E. coli* IMI was found in heifers supplemented with Se before calving (Table 6.2).

Finally, a single injection of barium selenate before calving may provide a fast release of Se from the site of injection shortly after supplementation, but that release may not be constant in a long-term study (Mallinson et al., 1985; Grace et al., 2001). Therefore, the release of Se from the site of injection could be lower at the end of the study period than at the beginning, which translates into a lower contribution to the equivalent dietary Se intake at the end of lactation, when the mammary gland is at higher risk of infection.

7.5   Conclusions

A summary of the published evidence about oral Se supplementation and its effect on milk Se concentration was presented in this thesis. In addition, the results described in this thesis provide an insight into the association of Se status on measures of udder health of cows in selected Atlantic Canadian dairy herds, and selected Canadian herds, and on the effect of Se supplementation on udder health in dairy cattle under pastoral dairy systems.

The main conclusions of this thesis are:

i. Oral supplementation in cattle increases milk Se concentration, with a high between-study variance due in part to geographic factors, and study design characteristics (e.g. Se source and rate of Se supplementation) (Chapter 2).

ii. Organic Se was associated with a greater increase in milk Se than an equal mass of inorganic dietary Se (Chapter 2).

iii. Individual milk Se concentration and BTSe are reflecting an adequate Se status in selected Canadian dairy herds. Likely, these adequate values are the result of widespread improvements in the practice of supplementing Se in those particular herds (Chapter 3).

iv. A higher milk Se concentration is associated with higher SCC in milk. However, milk Se concentration does not appear to be a determinant of udder health in selected cows from Atlantic Canada.

v. Bulk tank milk Se in Canadian dairy herds is not associated with milk production parameters or BTSCC. However, higher values of BTSe are associated with lower risk of being a *Staph. aureus*-positive herd. Likely, an .adequate Se status in Canadian herds resulted into a more robust udder immune response to contagious mammary pathogens (Chapter 4).

vi. Lower dietary Se intake appears to be enough to prevent subclinical mastitis in pasture-based cows (Chapter 5), but pre-calving Se supplementation in first-calving heifers grazing areas with low Se pastures in Southern Chile, regardless of Se, is beneficial for preventing subclinical mastitis shortly after at calving (Chapter 6).

Despite the considerable literature concerning Se nutrition in dairy cattle, including that presented in this thesis, it seems unlikely that we will be able to make general recommendations for optimal Se supplementation practices that will be applicable across all management systems.

However, despite the differences between supplementation practices in Canadian and Chilean herds, current Se supplementation practices being employed in these countries appear to be adequate to ensure udder health in most situations. Nonetheless, two specific recommendations regarding strategic supplementation to improve udder health can be made from the work findings presented herein: the prevalence of *Staph. aureus* in Canadian herds may be reduced as the Se content in milk is increased, and heifers on pasture may benefit from strategic increase in Se administration prior to calving to prevent IMI during the periparturient period.

7.6   Future Research on Selenium Supplementation and Mastitis

According to the results obtained in this thesis, several aspects of Se supplementation in dairy cattle require further investigation. A few questions were identified and are still waiting to be answered.

The evaluation of Se status should require the determination of other markers concomitantly, such as other selenoproteins and blood or plasma Se concentration, and not just milk Se concentration or blood GPx activity. The latter is not the only cytoprotective selenoprotein. Approximately 25 selenoproteins have been identified to date (Hatfield et al., 2006), with some of them acting as antioxidants and directly involved in the protection of the udder against pathogens (Sordillo et al., 2007; Sordillo and Aitken, 2009). Therefore, the actual measurement of selenoprotein activity in PMN would help to determine the overall effect of Se status on known protective mechanisms, addressing some of the questions about the cytoprotective effect of Se and its role in the immune response of the mammary gland (Sordillo, 2009. Personal communication)[6].

The selection of the biomarkers to evaluate the Se status or the response to Se supplementation requires additional consideration regarding the duration of the study. Plasma or serum Se should be considered in short-term trials, while whole blood Se concentration and the concentration of specific selenoproteins would be better for measuring the response to supplementation in long-term trials.

The positive association of milk Se concentration with the risk of having a new *Streptococcus* spp. IMI or other Gram-positive IMI in cows from Atlantic Canadian herds (Chapter 3) suggests a pathogen-specific effect; additionally, mastitis-causing pathogens might be different according to the dairy production system. More work is needed to understand the role of Se in differences of the udder immune response against pathogens, such as *Staph. aureus*, *Streptococcus* spp., and CNS. A systematic review and meta-analysis on the effect of Se on udder health would help to address these questions, as they would allow summarizing the effect of the intervention (e.g. Se status or supplementation) and evaluate the potential sources of heterogeneity (e.g. dairy production system).

Few trials have examined the effect of organic Se (e.g. Se yeast or Se-enhanced forages and crops) on the dynamics of IMI and SCC under commercial conditions of dairy farming. Malbe et al. (1995) reported no evidence for organic Se over inorganic sources in improving the udder health in supplemented cows. Selenium supplementation before calving tended to decrease the prevalence of IMI and SCC at calving irrespective of the Se source (Chapter 6), but organic Se may be superior to inorganic sources in terms of its bioavailability (Weiss, 2005) and the effect on milk Se concentration (Chapter 2). However, higher milk Se concentration does not necessarily mean higher incorporation into functional selenoproteins. The role of organic sources and their incorporation into functional selenoproteins, particularly into those involved in the udder immune response, requires more research.

Finally, the effect of potential confounders of the response to Se supplementation, such as Se status before the beginning of the study (i.e. in supplementation trials), the status of vitamin E and its concentration in the ration, and the diet concentration of PUFA, should be considered in any observational or interventional trial about the effect of Se on the health status of dairy cattle.

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APPENDIX 1

List of references excluded from the meta-analysis (Chapter 2)

Reason for exclusion	Reference
Not clinical trials	Conrad y Moxon, 1980
	Moxon y Palmquist, 1980
	Symonds et al., 1981
	Binnerts et al., 1984
	Ekholm et al., 1991
	Pehrson and Ortman, 1995
	Pehrson et al., 1997
	McDowell, 2002
	Serdaru et al., 2004
	Walker et al., 2004
	Pehrson, 2005
	Weiss, 2005
Did not report the outcome of interest	Dell, 1974
	Hidiroglou et al., 1977
	Lein et al., 1980
	Pehrson and Johnson, 1985
	Fraser et al., 1987
	Tasker et al., 1987
	Kincaid and Hodgson, 1989
	Eversole et al., 1992
	Coe et al., 1993
	Kohler et al., 1994
	Charlton, 1995
	Klawonn et al., 1996
	Lacetera et al., 1996
	Culleton et al., 1997
	Judson et al., 1997
	Acevedo-Maldonado et al., 1999
	Enjalbert et al., 1999
	Falkowska et al., 2000
	Meglia et al., 2001
	Malbe et al., 2003
	Paula-Lopes et al., 2003
	Lewis, 2004
	Fan et al., 2006
	Harrison and Elliot, 2006
Reported duplicate data of other studies	Maus et al., 1978
	Nicholson et al., 1990
	Cuesta, 1992
	Fisher, 1995

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	Fisher et al., 1995
	Kessler and de Faria, 1998a
	Muniz-Naveiro et al., 2006
	Muniz-Naveiro et al., 2007
	Pehrson and Ortman, 1995
Described results of parenteral Se supplementation	Little et al., 1979
	Schingoethe et al., 1982
	De Toledo and Perry, 1985
	Hidiroglou et al., 1987[a]
	Culleton et al., 1993
	Cuesta et al., 1994
	Zarski and Debski, 1996
	Grace et al., 1997
	McDowell et al., 2002
	Pavlata et al., 2003
	Pavlata et al., 2004
Control group was not unsupplemented	Maus et al., 1980
	Essick and Lisk, 1987
	Fisher et al., 1995
	Ortman and Pehrson, 1997
	Awadeh et al., 1998
	Kessler and de Faria, 1998b
	Grundler, 1999
	Pehrson et al., 1999
	Ivancic and Weiss, 2001
	Pehrson and Arnesson, 2003
	Brzoska, 2004
	Foltys et al., 2004
	Givens et al., 2004
	Lewis, 2004
	Diaz et al., 2005
	Harrison et al., 2005a,b
	Weiss and Hogan, 2005
	Wiewiora et al., 2004
Two sources of Se given to the same animal	Koller et al., 1984
Two routes of Se administration to the same animal	Salih et al., 1987

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Breakdown by herd of the cows lost to follow up (Chapter 3)

Province[1]	Herd	n[1]	Lost[2]	Reason[3]
New Brunswick	1	15	0	-
	2	16	1	No calving
	3	15	0	-
	4	16	4	No calving
	5	17	4	No samples
Nova Scotia	6	15	0	-
	7	15	0	-
	8	19	1	No calving
	9	13	1	No samples
	10	13	3	No samples
Prince Edward Island	11	11	1	No samples
	12	15	0	-
	13	15	15	Left study
	14	15	2	No calving
	15	15	1	No samples
	16	14	0	-
	17	8	8	Left study
	18	15	2	No calving
		262	43	

[1]Cows enrolled at the beginning of the study.
[2]Cows lost to follow after drying-off, Se determined at drying-off.
[3]No calving: cow(s) that did not calve during the study period, No samples = no samples collected after calving, Left study: farm left the study.

APPENDIX 3

Breakdown by region and season of unavailable samples for the evaluation of bulk tank milk selenium concentration in Canadian dairy herds

Region	Season	Available	Unavailable
Western Canada	Winter	7	8
	Spring	6	9
	Summer	7	8
	Fall	0	15
Ontario/Québec	Winter	28	3
	Spring	22	6
	Summer	2	29
	Fall	0	31
Atlantic Provinces	Winter	11	6
	Spring	17	0
	Summer	15	2
	Fall	6	11

APPENDIX 4

Breakdown by treatment group and days of the lost quarter samples due to the fire of the laboratory of microbiology (Chapter 6)

Quarter	Days	Treatment		
		Unsupplemented	Barium selenate	Se yeast
Right front	0-1	26	40	20
	7-28	106	115	74
Right rear	0-1	24	40	20
	7-28	105	111	73
Left front	0-1	24	40	20
	7-28	105	111	79
Left rear	0-1	26	40	20
	7-28	105	115	75
Total		521	612	381