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INGER JOHANNE KARLENGEN

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EFFECT OF COBALT ON FATTY ACID COMPOSITION IN MILK OF DAIRY COWS AND SOWS

EFFEKT AV KOBOLT PÅ FETTSYRESAMMENSETNINGEN I MELK HOS MELKEKYR OG
PURKER

INGER JOHANNE KARLENGEN

Effect of cobalt on fatty acid composition in milk of dairy cows and sows

Effekt av kobolt på fettsyresammensetningen i melk hos melkekyr og purker

Philosophiae Doctor (PhD) Thesis

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Inger Johanne Karlengen

ABSTRACT

Karlengen, I.J. 2011. Effect of cobalt on fatty acid composition in milk of dairy cows and sows. Norwegian University of Life Sciences, Philosophiae Doctor (PhD) Thesis 2011:75, ISSN 1503-1667, ISBN 978-82-575-1038-1.

Previous research revealed that supplementing excess cobalt (Co) reduces bovine milk content of monounsaturated fatty acids (MUFAs), probably mediated by the enzyme $\Delta 9$ -desaturase. The general aim of this research project was to further explore the effect of Co on the desaturation of fatty acids (FAs), especially in lactating dairy cows, but also in sows. Three experiments were performed. In the first study, the main subject was to investigate the effects of excess Co on FA composition of blood plasma and milk from lactating sows (Paper I). Experimental animals were 10 lactating sows: five intramuscularly injected with saline solution (control) twice a day, and five intramuscularly injected with a Co solution twice a day (118 mg Co/d). The treatment lasted five days. Fatty acid composition of blood plasma and milk fat was determined. Cobalt had no effect on FA composition of blood plasma. In contrast, milk fat from the sows receiving Co showed a significant ($p < 0.05$) decrease in the concentration of myristoleic acid (*cis*-9 14:1) and palmitoleic acid (*cis*-9 16:1), confirming that excess Co also lowered the $\Delta 9$ -desaturation of milk fat from lactating sows.

In study 2, the objective was to determine the effect of excess Co on gene expression levels of $\Delta 9$ -desaturase, FA synthase (FASN), diacylglycerol acyltransferase 1 (DGAT1) and diacylglycerol acyltransferase 2 (DGAT2) in the mammary gland of lactating dairy cows (Paper II). Seven cows were given 1.4 g Co *per os* twice daily for seven days, followed by a nine-day depuration period. Udder biopsies were performed three times during the experiment: before the treatment started, at the end of the treatment period, and at the end of the depuration period. Gene expression levels of $\Delta 9$ -desaturase, FASN, DGAT1 and DGAT2, and FA composition of milk fat were determined. Excess Co significantly reduced ($p < 0.05$) the concentration of all *cis*-9 MUFAs in milk fat, confirming a reduction in $\Delta 9$ -desaturation in the mammary gland. However, neither $\Delta 9$ -desaturase, FASN, DGAT1 nor DGAT2 gene expression levels were significantly affected, indicating that the effect of Co on milk FA profile observed was mediated by a reduction in $\Delta 9$ -desaturase activity in the mammary gland at the post-transcriptional level.

The third study was conducted to determine what amount of Co is needed to lower the proportion of *cis*-9 MUFAs in bovine milk fat (Paper III). Four lactating dairy cows were used in a 4 x 4 Latin square design study. The basal diet of grass silage and concentrate mixture was supplemented with four levels of Co: 0 mg/d, 4.0 mg/d, 360 mg/d, and 5100 mg/d. The Co solutions were continuously infused into the rumen for 11 days. Milk yield and milk content of fat, FAs, protein, lactose, Co, zinc (Zn), iron (Fe) and copper (Cu) were determined. Blood plasma was analyzed for contents of FAs, Co, Zn, Fe and Cu. Feed intake and total tract digestibility of the feed components were also determined. Cobalt affected the proportion of some FAs in blood plasma, but the effects were relatively small in magnitude. With few minor exceptions, only the highest level of Co (5100 mg/d) significantly lowered the MUFA proportion in milk fat. Oleic acid (*cis*-9 18:1) was reduced as much as 38% compared to that with the lowest Co level. However, the highest level of Co also lowered feed intake and milk yield.

The main conclusion from the present studies is that excess Co also lowers the proportion of MUFAs in sow's milk, and that this effect of Co in dairy cows is probably mediated by post-transcriptional reduction of Δ 9-desaturase activity. Moreover, the amount of Co needed to suppress Δ 9-desaturation in bovine milk is so high that the normal levels of Co in rations for dairy cows do not significantly affect milk FA composition.

SAMMENDRAG

Karlengen, I.J. 2011. Effekt av kobolt på fettsyresammensetningen i melk hos melkekyr og purker. Universitetet for miljø- og biovitenskap, PhD avhandling 2011:75, ISSN 1503-1667, ISBN 978-82-575-1038-1.

I tidligere forsøk med melkeku har tildeling av store mengder kobolt (Co) redusert innhold av enumettede fettsyrer i melka. Denne effekten ble forklart med at Co trolig virket negativt på enzymet $\Delta 9$ -desaturase. Hensikten med dette forskningsprosjektet var å belyse nærmere virkninger av Co på desaturering av fettsyrer, spesielt hos lakterende melkekyr, men også hos purker. Det ble gjennomført tre forsøk. Hovedhensikten med det første forsøket var å undersøke effekten av store mengder Co på fettsyresammensetningen i blodplasma og melk fra lakterende purker (Paper I). Ti lakterende purker ble brukt som forsøksdyr; fem fikk fysiologisk saltvann injisert intramuskulært to ganger daglig (kontroll), og fem fikk Co-løsning injisert intramuskulært to ganger daglig (118 mg Co/dag). Behandlingen pågikk i fem dager, og fettsyresammensetningen i blodplasma og melk ble bestemt. Kobolt hadde ingen effekt på fettsyresammensetningen i blodplasma, men hadde signifikant ($p < 0,05$) negativ virkning på konsentrasjonen av myristoleinsyre (*cis*-9 14:1) og palmitoleinsyre (*cis*-9 16:1) i melk. Disse resultatene bekreftet dermed at store mengder Co reduserte $\Delta 9$ -desaturering av melkefett også hos lakterende purker. I det andre forsøket var hensikten å undersøke effekten av store mengder Co på genekspresjonsnivået av $\Delta 9$ -desaturase, fatty acid synthase (FASN), diacylglycerol acyltransferase 1 (DGAT1) and diacylglycerol acyltransferase 2 (DGAT2) i juret hos lakterende melkekyr (Paper II). Sju kyr ble tildelt 1,4 g Co oralt to ganger daglig i sju dager, etterfulgt av en ni dagers utvaskingsperiode. Det ble tatt jurbiopsier tre ganger i løpet av forsøket; før behandlingen startet, i slutten av behandlingsperioden og på slutten av utvaskingsperioden. Genekspresjonsnivået av $\Delta 9$ -desaturase, FASN, DGAT1 og DGAT2, samt fettsyresammensetningen i melk ble bestemt. Cobalt reduserte konsentrasjonen av alle *cis*-9 enumettede fettsyrer i melkefett signifikant ($p < 0,05$). Dette bekrefter resultater oppnådd i tidligere forsøk om at store mengder Co reduserer $\Delta 9$ -desaturering av melkefettet. Imidlertid var ikke genekspresjonsnivået av hverken $\Delta 9$ -desaturase, FASN, DGAT1 eller DGAT2 signifikant påvirket. Dette indikerer at effekten av Co på fettsyresammensetningen i melk var forårsaket av en post transkripsjonal hemming av $\Delta 9$ -desaturase aktiviteten i juret.

Det tredje forsøket ble gjennomført for å undersøke hvor stor mengde Co som må til for å redusere andelen *cis-9* enumettede fettsyrer i melkefett fra kyr (Paper III). Fire lakterende kyr inngikk i et 4 x 4 Latinsk kvadrat. Grunnrasjonen bestående av grassurfôr og kraftfôr ble supplementert med fire ulike nivåer av Co; 0 mg/d, 4,0 mg/d, 360 mg/d, og 5100 mg/d. Kobolt løsningene ble kontinuerlig infundert i vomma i 11 dager. Melkeytelse ble målt, og melka ble analysert for innhold av fett, fettsyrer, protein, laktose, Co, sink (Zn), jern (Fe), og kobber (Cu). Blodplasma ble analysert for innhold av fettsyrer, Co, Zn, Fe og Cu. Fôropptak og totalfordøyelighet av fôret ble også bestemt. Kobolt påvirket andelen av noen fettsyrer i plasma negativt, men utslagene var relativt små. Med noen få unntak var det bare det høyeste Co nivået (5100 mg/d) som reduserte andelen enumettede fettsyrer i melka signifikant, og oljesyre (*cis-9* 18:1) ble redusert med så mye som 38% sammenlignet med det laveste Co nivået. Både fôropptaket og melkeytesen ble redusert med det høyeste Co nivået.

Hovedkonklusjonen fra disse forsøkene er at store mengder kobolt reduserer andelen enumettede fettsyrer også i melk fra purker, og at denne effekten av Co hos melkekyr antakelig skyldes redusert aktivitet av $\Delta 9$ -desaturase post transcriptionalt. Videre er mengden Co som er nødvendig for å hemme $\Delta 9$ -desaturering i juret så høy at de normale mengdene av Co i rasjonen til melkekyr ikke vil påvirke fettsyresammensetningen i melka.

ABBREVIATIONS

ACACA – acetyl-CoA carboxylase alpha

AGPAT – acyl glycerol phosphate acyl transferase

ChREBP – carbohydrate-response element-binding protein

Co – cobalt

DAG – diacylglycerol

DGAT – diacylglycerol acyl transferase

EDTA – ethylenediaminetetraacetic acid

ER – endoplasmatic reticulum

FA – fatty acid

FASN – fatty acid synthase

Fe – iron

GPAT – glycerol-3 phosphate acyl transferase

LPL – lipoprotein lipase

LXR – liver X receptor

MUFA – monounsaturated fatty acid

NEFA – non-esterified fatty acid

PL – phospholipid

PPAR – peroxisome proliferator activated receptor

PUFA – polyunsaturated fatty acid

SFA – saturated fatty acid

SREBP – sterol-response element-binding protein

TAG – triacylglycerol

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, and they are referred to in the text by their Roman numerals.

- I. Karlengen I. J., Harstad, O. M., Kjos, N. P., Salbu, B., Aastveit, A. H. and Taugbøl, O. 2011. Cobalt reduces the $\Delta 9$ -desaturase index of sow milk. *Journal of Animal Physiology and Animal Nutrition*. 95: 676-684.

- II. Karlengen I. J., Harstad, O. M., Taugbøl, O., Berget, I., Aastveit, A. H. and Våge, D. I. The effect of excess cobalt on milk fatty acid profiles and transcriptional regulation of SCD, FASN, DGAT1 and DGAT2 in the mammary gland of lactating dairy cows. *Journal of Animal Physiology and Animal Nutrition*. *In press*.

- III. Karlengen, I. J., Taugbøl, O., Salbu, B. and Harstad, O. M. Effect of different levels of supplied cobalt on the fatty acid composition of bovine milk. *British Journal of Nutrition*. *Submitted*.

1 BACKGROUND AND OBJECTIVES

Milk and milk products are important sources of energy and nutrients in human diets (Lawson et al., 2001, Weinberg et al., 2004, Ranganathan et al., 2005, Haug et al., 2007). Cow's milk contains 3.5 to 5% fat. Based on data from the United States, dairy products contribute as much as ~15% of the total fat in the human diet, and an even greater part of the total saturated fat (~25%) (O'Donnell, 1993, Williams, 2000). In northern Europe, consumption of dairy products is higher than in the United States (IDF, 2007). This relatively high intake of saturated fatty acids (SFAs) from milk fat has led to a public health concern. Especially myristic- (14:0) and palmitic (16:0) acids are reported to have negative effects, whereas stearic acid (18:0) is reported to be neutral in its effects on human health (Haug et al., 2007). Generally, diets high in mono unsaturated fatty acids (MUFAs) lower plasma cholesterol, LDL-cholesterol and triacylglycerol concentrations (Kris-Etherton et al., 1999). In addition, replacing SFAs with *cis*-unsaturated fatty acids have been shown to lower the risk for coronary artery disease (Mensink et al., 2003), indicating that an increase in *cis*-9 MUFAs and a decrease in SFAs is desirable in milk fat. Among the main components of milk, fat is the most modifiable (Chilliard et al., 2000, Haug et al., 2007). Therefore, the impact of dairy cow nutrition on fatty acid (FA) composition is of great interest, and has been extensively reviewed (Sutton, 1989, Grummer, 1991, Palmquist et al., 1993, Kennelly, 1996, Ashes et al., 1997, Mansbridge and Blake, 1997, Chilliard et al., 2000, Jensen, 2002, Chilliard and Ferlay, 2004).

In 2003, results obtained in our department indicated that the concentration of MUFAs was lower in milk from dairy cows supplied with cobalt-ethylenediaminetetraacetic acid (Co-EDTA) and Ytterbium-acetate (Yb-acetate) as digestion markers (unpublished results). These indications were supported by results obtained in digestion studies with dairy cows using the triple marker system comprising Co-EDTA, Yb-acetate and chromium-mordanted straw in Finland, and it was suggested that the effect was probably related to Δ^9 -desaturase, the rate-limiting enzyme of MUFA synthesis (Shingfield et al., 2006). Follow up studies in Finland (Shingfield et al., 2008) revealed that this effect was caused by the marker Co-EDTA. Recently, it was shown in our department that cobalt (Co) alone was responsible for the reduction in the proportion of MUFAs in milk fat (Taugbøl et al., 2008). Follow up studies revealed that the effect of Co on MUFAs in milk fat occurred after absorption from the digestive tract (Taugbøl et al., 2010).

Besides the decisive impact of $\Delta 9$ -desaturase on the proportion of MUFAs in milk fat, the products of $\Delta 9$ -desaturase are major substrates for the synthesis of various types of lipids, such as phospholipids (PLs), triacylglycerols (TAGs) and cholesterol esters (Nakamura and Nara, 2004). Alteration of the ratio between SFAs and MUFAs in PLs has been implicated in overall energy metabolism and a variety of disease states (Ntambi and Miyazaki, 2004). Accordingly, $\Delta 9$ -desaturase plays a key role in fat metabolism in general across species. However, information about the effect of Co on $\Delta 9$ -desaturation in non-ruminants is scarce. Therefore, experiments to determine if this effect of Co on $\Delta 9$ -desaturase is general or specific for ruminants were of great interest. Even though the present effect obtained of excess Co on bovine milk fat composition was undesirable from a human health perspective, further research was important for different reasons. In the first instance, it was of decisive importance to determine how much Co is needed to obtain the effect on the proportion of MUFA in bovine milk, and whether the commonly used level of Co in the diets of dairy cows has an effect. Secondly, more knowledge about the mechanism behind the effects was important and a necessary platform for making progress with the aim of obtaining the opposite and positive impact on milk FA composition. From a theoretical point of view, Co could reduce $\Delta 9$ -desaturation by lowering the gene expression levels of the enzyme, the activity of the enzyme *per se*, or both (Miyazaki and Ntambi, 2008). It was important to conduct a more in-depth study of the mechanisms responsible for the effect of Co on the desaturation of SFAs in bovine milk.

Based on this background, the following objectives were stated:

1. To examine the effect of excess Co on the proportion of MUFAs in blood plasma and milk of sows.
2. To examine if the lowering effect of excess Co on MUFAs in bovine milk fat is caused by reduction in gene expression of $\Delta 9$ -desaturase.
3. To see if the level of Co commonly used in dairy cow rations negatively affects milk FA composition, and to determine the level of Co that is needed to influence bovine milk FA composition.

2 GENERAL INTRODUCTION

2.1 About cobalt

Cobalt has atomic number 27, and a molar mass of 58.93 g/mol. It is a transition metal and belongs to the same family as iron (Fe) and nickel in the periodic table. Cobalt and Fe have similar physical and physiological properties such as valences (+2, +3) and transferrin binding (Smith, 2005, Chikh et al., 2008).

2.1.1 Cobalt in feed

The concentration of Co in plant material is dependent on several factors, such as soil content of Co, soil pH, and plant species (Mills, 1981). Uptake of soil Co by forages decreases as pH increases, and alkaline soils or liming of soils can prevent adequate uptake of Co by plants (Mills, 1981). Most feedstuffs are low in Co, containing less than 0.5 mg/kg DM (EFSA, 2009). Accordingly, due to local occurrence of soils deficient in Co, the forages may not meet the animal requirements. Such areas deficient in Co have been reported in Australia, New Zealand, East Africa, Norway and throughout Central and South America (Ammerman and Goodrich, 1983).

2.1.2 Cobalt metabolism

Cobalt is an essential trace element for ruminants because it is a component of vitamin B12, which is synthesised in the digestive tract by microbial action (NRC, 1980). The incorporation rate of Co into vitamin B12 in ruminants ranges between 3 and 15%, inversely related to Co intake (Smith and Marston, 1970, Stemme et al., 2008, Girard et al., 2009). As dietary Co increases, the microbes also produce a number of analogues of vitamin B12, which are not physiologically active (Halpin et al., 1984). The absorption of vitamin B12 occurs primarily from the small intestine (Rerat et al., 1956, Rerat et al., 1958a, Smith and Marston, 1970), but the rumen is permeable to high concentrations of vitamin B12 in the free form (Rerat et al., 1958b).

Absorption of inorganic Co also occurs in the small intestine, and during absorption, Co is known to interact with Fe (Thomson et al., 1971, Flanagan et al., 1980, McDowell, 2003).

Cobalt and Fe appear to share a common intestinal transport system, at least in monogastric mammals (Thomson et al., 1971). The absorption mechanisms of inorganic Co in ruminants seem to be poorly investigated, but it was found that the extent of absorption is very low (Barnaby et al., 1968, Hollins and McCullough, 1971, Looney et al., 1976, Vanbruwaene et al., 1984, Ayala-Fierro et al., 1999). Looney et al. (1976) estimated that as much as 95 to 98% of ⁶⁰Co given orally to sheep was excreted via the faeces within five days, whereas only 0.5 to 2.0% was excreted via the urine.

Cobalt that is absorbed from the digestive tract follows aqueous excretion routes via the urine, and also via milk (Vanbruwaene et al., 1984). Studies with various animal species (Table 1) show that more than half of Co injected intravenously is excreted during the first 24 h and more than two-thirds is excreted during the first week (Bailey et al., 1989). Most of the Co injected intravenously is excreted in the urine (Table 1), but small amounts (4-28% of the injected Co over the first week) appear to be recycled into the digestive tract and excreted in the faeces (Andre et al., 1989, Bailey et al., 1989, Collier et al., 1989, Talbot and Morgan, 1989, Kirchgessner et al., 1994).

Table 1 Cumulative urinary and faecal excretion of cobalt following intravenous injection of Co(NO₂)₃ (after Bailey et al., 1989)

	Cumulative excretion of cobalt (%)					
	After 1 d		After 7 d		After 21 d	
	Urine	Faeces	Urine	Faeces	Urine	Faeces
Baboon	57	5	74	17	80	20
Beagle dog	71	3.4	86	4.4	87	4.9
Guinea pig	64	2.2	82	10	85	12
Rat	64	18	72	24	74	24
Hamster	55	17	68	28	69	29
Mouse	59	18	71	26	72	27

The only known essential function for Co is serving as a component of vitamin B12 (EFSA, 2009). However, it is reported that Co ions can replace other minerals to activate several enzyme systems and that Co ions have a function in haematopoiesis, that is independent of

vitamin B12 activity (Henry et al., 1997). Cobalt may also play a role in rumen fermentation by increasing the digestion of low quality forages (Lopez-Guisa and Satter, 1992, Zelenak et al., 1992). This effect may be due to the selection of certain microbial populations with a higher Co requirement or may be the result of the divalent Co cation forming crosslinks between negatively charged forage particles, which allows bacteria to cling to forage particles more efficiently (Lopez-Guisa and Satter, 1992).

2.1.3 Cobalt requirement and supply

Common signs of Co or vitamin B12 deficiency in animals are lack of appetite, reduction in body weight gain, feed intake and feed conversion, lack of thrift, severe emaciation, weakness, monoblastic anaemia, decreased fertility, and decreased milk and wool production (McDowell, 2003). Ruminants appear to be more sensitive to vitamin B12 deficiency than non-ruminants. This is largely because they are so dependent on gluconeogenesis for meeting needs of tissues for glucose. In addition, propionate-producing bacteria are dependent on vitamin B12 to produce propionate from succinate (Chen and Wolin, 1981, Strobel, 1992). The breakdown in propionate metabolism at the point where methylmalonyl-CoA is converted to succinyl-CoA may be a primary defect arising from a deficiency of vitamin B12 (Gawthorn et al., 1971). Without Co in the diet, production of vitamin B12 in the rumen declines within days (Underwood, 1981). On the contrary, stores of vitamin B12 in the liver of adult ruminants are usually sufficient to last for several months (Underwood, 1981). Accordingly, the cow may have adequate stores of vitamin B12 to last for several months, but the ruminal microbes do not. Therefore, ruminal concentrations of succinate will rise within a few days if the diet is deficient in Co (Kennedy et al., 1996). Based on estimates by Ammerman (1970) and Smith and Loosli (1957), the dietary requirement of Co for ruminants is set at 0.11 mg/kg diet DM (NRC, 2001). Even though most feedstuffs normally contain more Co than the requirement (NRC, 2001), it is common practise to supply dairy cows with extra Co as an insurance to cover the need in situation with feeds deficient in Co.

2.1.4 Toxicity of cobalt

Some of the physiological effects of high Co-levels that probably contribute to toxicological signs in animals are the dual effects on haem metabolism: High levels of Co enhance

erythropoietin synthesis through a complex mechanism that results in polycythaemia (Goldwasser et al., 1958), and induce the synthesis of haem oxygenase, the rate-limiting enzyme of haem degradation in liver, kidney and other tissues (Maines and Kappas, 1974, Maines and Kappas, 1975, Maines and Kappas, 1976b, Maines and Kappas, 1976a) (Llesuy and Tomaro, 1994). In ruminants, Co toxicity causes reduced feed intake, loss of body weight, hyperchromemia, and eventually anaemia (Ely et al., 1948, Keener et al., 1949, NRC, 1980). Little is known about the level Co that is toxic in adult cows. However, NRC (2005) set the maximum tolerable level for cattle to 25 mg/kg feed DM.

2.1.5 Cobalt as a digestion marker

In digestion experiments, Co-EDTA has been extensively used as a liquid phase marker in ruminants, and also in a variety of other animal species, including pigs (Turlington et al., 1989, Li et al., 1990, Jongbloed et al., 1992, Mroz et al., 1994, Corl et al., 2008). However, it is known that about 3 % of Co administered as Co-EDTA is recovered in the urine of ruminants (Uden et al., 1980), confirming that Co given as Co-EDTA is not completely indigestible. Because excess Co affects fat metabolism in dairy cows (Shingfield et al., 2006, Shingfield et al., 2008, Taugbøl et al., 2008), Co-EDTA is unsuitable as a liquid phase marker in studies including lipid metabolism and milk FA composition in ruminants. To our knowledge, no data are available on the excretion of Co-EDTA in pigs. At present, it is therefore uncertain if Co-EDTA is a suitable marker or not in studies with pigs where fat metabolism is involved.

2.2 Milk fat

Mammary epithelial cells of lactating animals are highly active in the biosynthesis of TAG. An average mammal produces 1-2 ml of milk per gram of mammary tissue per day, with a fat content that ranges between species from around 2 g/l to around 600 g/l (Clegg et al., 2001). The dairy cow produces milk with a fat content of 35 to 50 g/l. Of milk fat, TAGs constitute more than 95%, and the residual fat components are PLs, cholesterol, cholesterol esters, diacylglycerol (DAG), monoacylglycerol and free FAs (Sheffy et al., 1952, Bernard et al., 2008).

Bovine milk fat has a high proportion of medium-chain FAs (C8:0 to C12:0), as is also the case with milk from humans and most other non-ruminant mammals. Characteristic for bovine milk fat is the presence of short chain FAs (4:0 and 6:0) (Dils, 1986). In addition, cow's milk fat contains a considerable amount of 14:0, and only about 60% FAs with sixteen or eighteen carbon atoms (Table 2). Linoleic acid (*cis*-9, 12 18:2) and linolenic acid (*cis*-9, 12, 15 18:3) are the major poly unsaturated FAs (PUFAs), and the proportions of *cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3 are close to each other.

Table 2 Normal content of major fatty acids in bovine milk

Fatty acids (common name)	Average range (wt%)
<i>Saturated fatty acids:</i>	
4:0 (butyric)	2-5
6:0 (caproic)	1-5
8:0 (caprylic)	1-3
10:0 (capric)	2-4
12:0 (lauric)	2-5
14:0 (myristic)	8-14
15:0 (pentadecanoic)	1-2
16:0 (palmitic)	22-35
17:0 (margaric)	0.5-1.5
18:0 (stearic)	9-14
<i>Unsaturated fatty acids:</i>	
<i>Cis</i> -9 16:1 (palmitoleic)	1-3
<i>Cis</i> -9 18:1 (oleic acid)	20-30
<i>Cis</i> -9,12 18:2 (linoleic acid)	1-3
<i>Cis</i> -9,12,15 18:3 (linolenic acid)	0.5-2.0

Source: Kaylegian and Lindsey (1995).

In sow's milk, the fat content is slightly higher compared to cow's milk, normally 5 to 7% (Klobasa et al., 1987). Sow's milk also differs from cow's milk and milk from most other non-ruminant mammals by containing very low quantities of short- and medium-chain FAs (Barry, 1964, Linzell et al., 1967, Rook and Witter, 1968), and a high quantity of *cis*-9 16:1 (Rook and Witter, 1968). Consequently, the FAs of sow's milk fat consist of as much as ~90% FAs with sixteen or eighteen carbon atoms (Rook and Witter, 1968). Linoleic acid and *cis*-9, 12, 15 18:3 are also the major PUFAs in sow's milk. However, the proportion of *cis*-9, 12 18:2 in sow's milk is high, where it is close to 10% of total FAs. Accordingly, the proportion of *cis*-9, 12 18:2 is more than 10 times the proportion of *cis*-9, 12, 15 18:3.

2.2.1 Milk fat synthesis in ruminants

Milk fat is either synthesised *de novo* in the mammary gland, or it is synthesised from FAs which are taken up from the blood (Figure 1) (Chilliard et al., 2000).

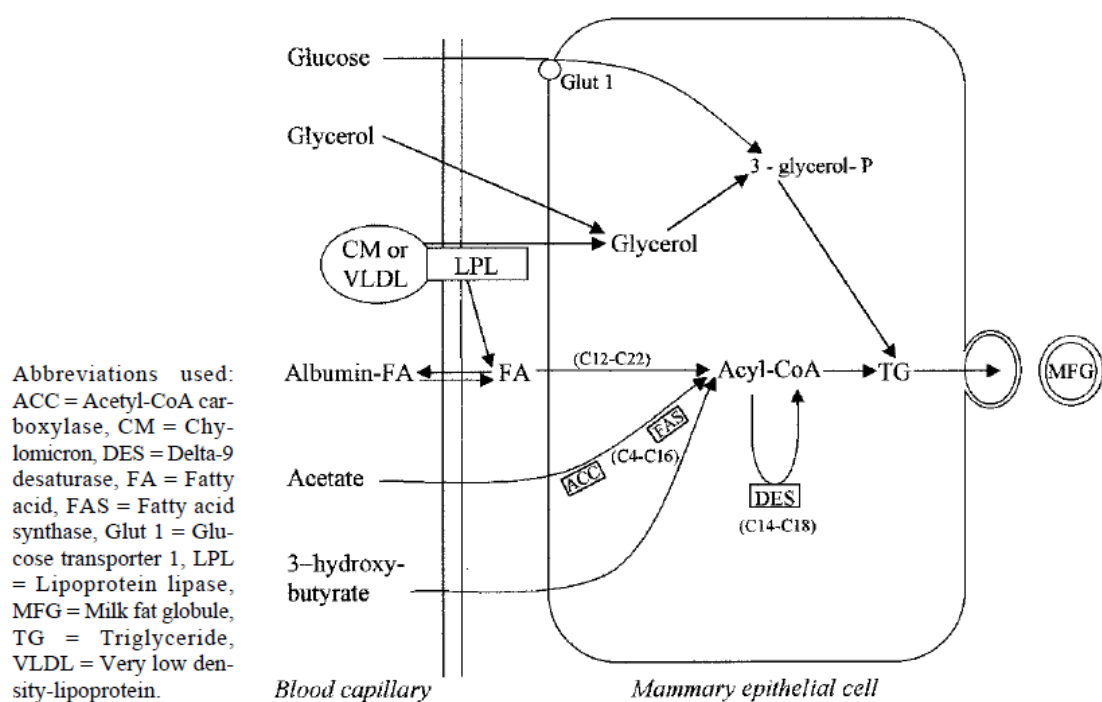


Figure 1 Milk fat synthesis and secretion in ruminants (Chilliard et al., 2000)

2.2.1.1 Supply of fatty acids to the udder

In ruminants, about 60% of the FAs secreted in milk are taken up from the blood (Chilliard et al., 2000). They originate from two sources; feed and adipose tissue. The fat fraction in ruminant diet normally contains a high proportion of unsaturated FAs, but they undergo total or partial hydrogenation in the rumen, resulting in a high proportion of SFAs absorbed from the digestive tract (Chilliard et al., 2000). Lipids originating from the feed and microbes are broken down to FAs and monoacyl glycerols in the small intestine before they are absorbed and reesterified to TAGs in epithelial cells. Inside the epithelial cells, TAGs form chylomicrons that are excreted into the blood via lymph vessels. Fatty acids from the chylomicrons are released by the enzyme lipoprotein lipase (LPL) before they are taken up by the mammary epithelial cells (Barber et al., 1997, Bernard et al., 2008). Mobilization of body fat occurs, especially during the period of negative energy balance in the beginning of lactation (Bauman and Griinari, 2001). According to the same cited authors, mobilization of body fat usually accounts for less than 10% of milk FAs, but this proportion increases in ruminants in negative energy balance in direct proportion to the extent of the energy deficit. Fatty acids mobilized from adipose tissue are transported in the bloodstream to the mammary gland as nonesterified FAs (NEFA) bound to albumin. Once the FAs reach the mammary gland, they are released from albumin and taken up by the mammary epithelial cells (Barber et al., 1997, Bernard et al., 2008).

Ruminants express several metabolic peculiarities to decrease the melting point of their body lipids, and especially milk lipids. These peculiarities include the synthesis of short- and medium-chain FAs, lack of chain elongation, desaturation of long-chain FAs (by intestinal, adipose and mammary tissue), and the uneven esterification pattern of the various FA molecules in mammary secretory cells (Chilliard et al., 2000).

2.2.1.2 De novo fatty acid synthesis

Short- and medium-chain FAs are synthesised *de novo* within mammary epithelial cells of ruminants, and consequently, the proportion of these FAs in milk reflects the contribution of mammary FA synthesis to total milk fat content (Barber et al., 1997). Normally, *de novo* synthesis contributes to about 40% of milk FAs. In ruminants, FAs are synthesized in the mammary gland from acetate and β -hydroxybutyrate supplied by the blood mainly from the rumen (Figure 1). β -Hydroxybutyrate contributes to about 15% of the carbon content of *de novo* synthesised FAs (Chilliard et al., 2000). Acetate and β -hydroxybutyrate contribute

almost equally to the initial four-carbon units of the FAs synthesised by the ruminant mammary tissues. (Palmquist et al., 1969).

Production of short chain FAs is under the control of acetyl-CoA carboxylase alpha (ACACA) which is believed to catalyse the rate-limiting step in *de novo* FA synthesis (Bauman and Davis, 1974), and the expression of ACACA is considerably upregulated in the mammary gland during lactation (Figure 2) (Bionaz and Loor, 2008). In subsequent steps, both acetyl-CoA and butyryl-CoA are primers for FA synthase (FASN), a cytosolic multifunctional protein (Palmquist, 2006), which is also upregulated in the mammary gland during lactation (Figure 2) (Bionaz and Loor, 2008). The major product of FASN is 16:0, but in ruminants, the enzyme also produces shorter chain FAs, down to 4 carbon atoms (Palmquist, 2006). Alteration of the specificity of the chain-termination reaction of FASN to produce medium-chain FAs in mammary tissue appears to be fundamentally different in ruminants and non-ruminants (Barber et al., 1997). The ruminant mammary gland contains an enzyme system (FASN) that is able to both load and release acyl chains from two- to 12-carbon chain lengths such that they can equilibrate between CoA-esters and enzyme-bound forms, independent of a thioesterase II (Grunnet and Knudsen, 1979, Grunnet and Knudsen, 1981, Knudsen and Grunnet, 1982, Mikkelsen et al., 1985). Thus, this synthesis in the mammary gland results in the short- and medium-chain FAs (C4:0 to C16:0) in milk (Chilliard et al., 2000). Another peculiarity in ruminants is the lack of availability to elongate 16:0 in the mammary gland (Annison et al., 1967, Bines and Brown, 1968). Therefore, all long-chain FAs (≥ 18 carbon-atoms) and about one-half of C16:0, depending on diet composition, originate from plasma FAs in ruminants (Bernard et al., 2008).

Milk from ruminants contains small amounts of odd- and branched-chain FAs. These FAs are largely derived from bacteria leaving the rumen, and it is suggested that variations in the profile of these FAs leaving the rumen are mainly a reflection of changes in the relative abundance of specific bacterial populations, rather than altered bacterial FA synthesis related to the availability of primers (Vlaeminck et al., 2006). Linear odd-chain FAs can also be synthesised *de novo* from propionate in adipose tissue and in the mammary gland of ruminants (Scaife et al., 1978, Dodds et al., 1981, Massartleen et al., 1983). High proportions of propionate in the rumen fluid are associated with large amounts of starch or soluble carbohydrates in the diet (Demeyer, 1981).

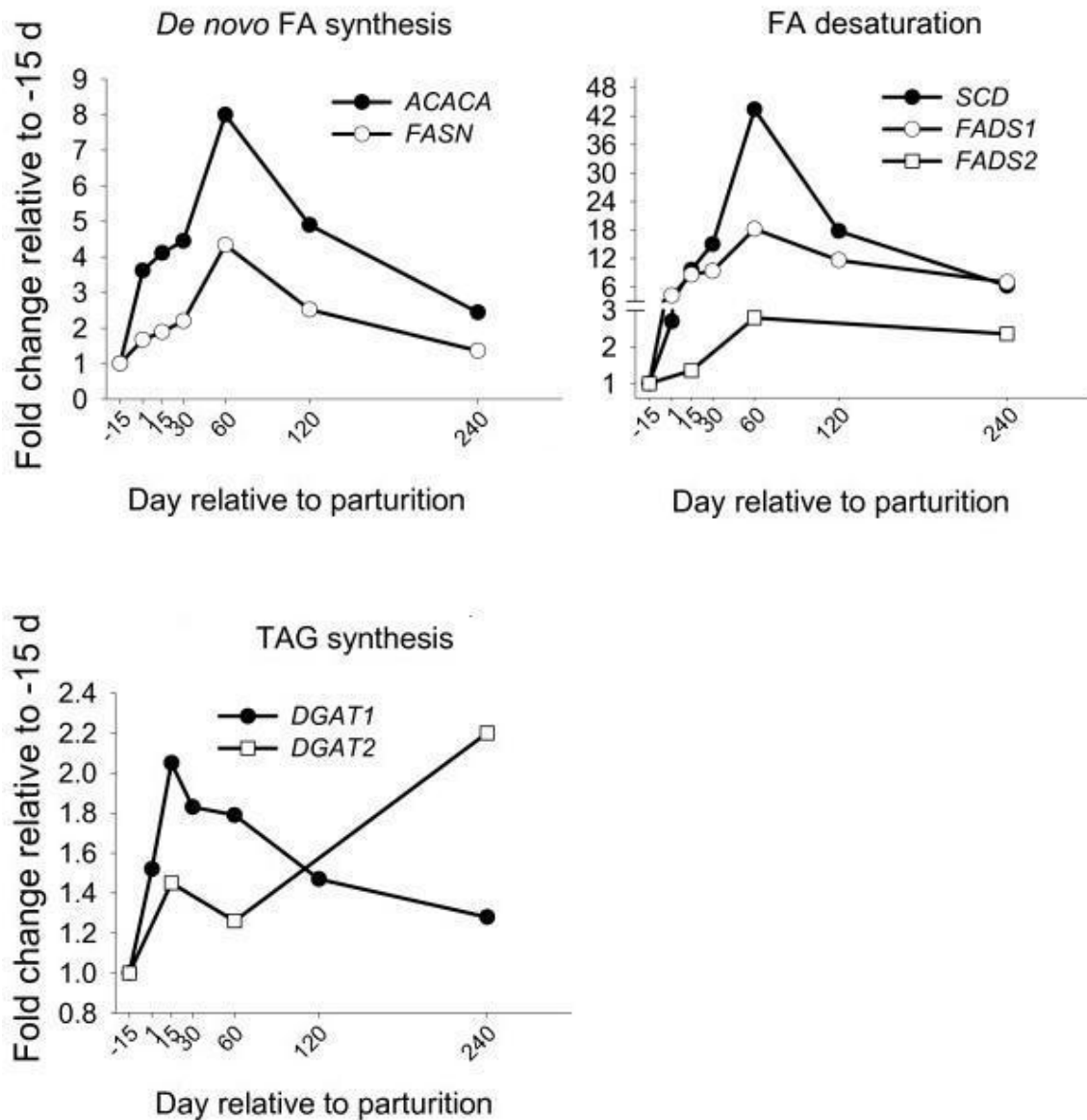


Figure 2. Temporal expression patterns in bovine mammary gland for genes involved in *de novo* FA synthesis, FA desaturation and TAG synthesis. SCD= Δ 9-desaturase; FADS1= Δ 5-desaturase; FADS2= Δ 6-desaturase. Adapted from Bionaz and Loor (2008)

2.2.1.3 Fatty acid desaturation

Fatty acid desaturases introduce a double bound at a specific position of FAs. The degree of desaturation affects physical properties of membrane PLs and stored TAGs (Nakamura and Nara, 2004), as well as physical and nutritional properties of milk fat. Delta 9-desaturase is the quantitatively most important desaturase involved in milk fat synthesis (Bionaz and Loor, 2008).

The enzyme $\Delta 9$ -desaturase is a microsomal membrane-bound protein and the rate-limiting enzyme catalysing the synthesis of MUFAs from SFAs (Nakamura and Nara, 2004, Paton and Ntambi, 2009). The enzyme introduces a *cis*-double bond at the 9, 10 position from the carboxyl end of FAs, and preferred substrates are 16:0 and 18:0, which are converted into *cis*-9 16:1 and *cis*-9 18:1, respectively (Ntambi, 1995). Fully differentiated ruminant mammary secretory cells express a high $\Delta 9$ -desaturase activity (Kinsella, 1972), and 18:0 desaturated in the mammary gland contributes to more than 50% of the *cis*-9 18:1 that is secreted into milk fat (Bickerstaffe et al., 1974, Enjalbert et al., 1998). The desaturation by $\Delta 9$ -desaturase is an oxidative reaction, involving cytochrome b₅, NADH cytochrome b₅ reductase and molecular oxygen, in addition to the Fe-containing enzyme $\Delta 9$ -desaturase (Figure 3) (Ntambi, 1999). Electron transport from NADH via FAD of NADH cytochrome b₅ reductase and the heme-Fe in the hydrophilic domain of cytochrome b₅ to the di-Fe complex of the desaturase is absolutely required for the desaturation process to take place (Broadwater et al., 1998).

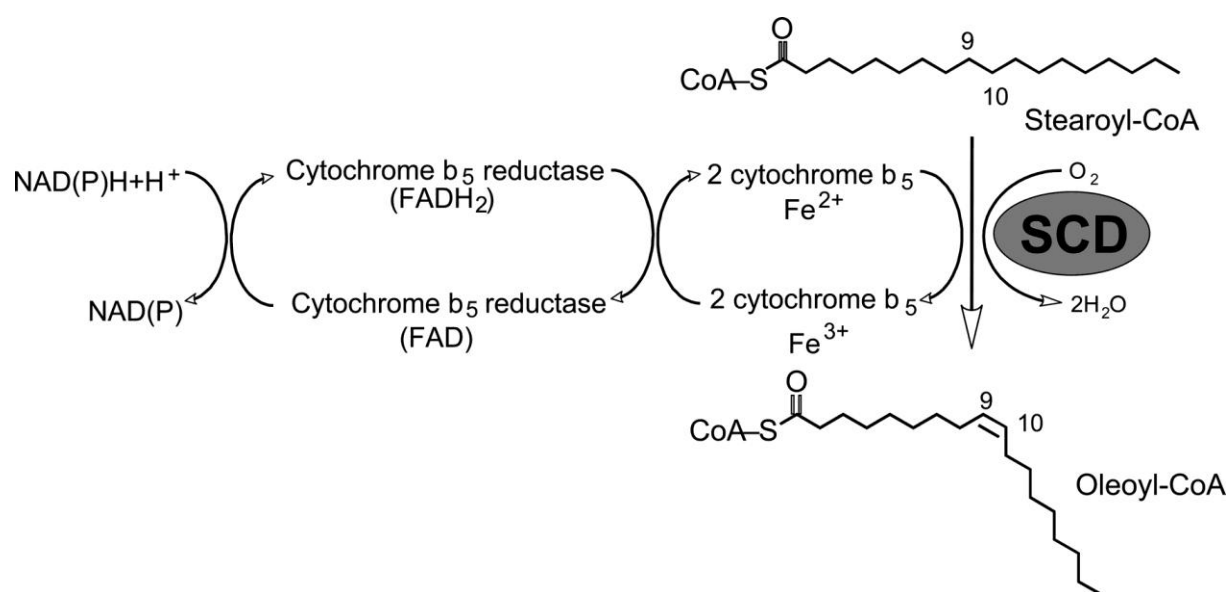


Figure 3 The pathway of electron transfer in the desaturation of fatty acids by SCD ($\Delta 9$ -desaturase) (Paton and Ntambi, 2009)

The mammary gland is a major site of $\Delta 9$ -desaturation in lactating ruminants, and $\Delta 9$ -desaturase is highly expressed in the mammary gland during lactation (Figure 2) (Bionaz and Looor, 2008).

Like $\Delta 9$ -desaturase, $\Delta 6$ -desaturase and $\Delta 5$ -desaturase are membrane-bound desaturases, found in the endoplasmic reticulum (ER) of animals (Pereira et al., 2003). The enzyme $\Delta 6$ -desaturase introduces a *cis*-double bond at the 6, 7 position from the carboxyl end of FAs and has an important role, where it is the rate-limiting step in the desaturation/elongation pathway of the 18-carbon essential FAs *cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3 to arachidonic acid (*cis*-5, 8, 11, 14 20:4) and docosahexaenoic acid (*cis*-5, 8, 11, 14, 17 20:5), respectively (Brenner, 1974). It is a non-haem Fe protein containing one atom of Fe per molecule of the enzyme. As with $\Delta 9$ -desaturase, it has been demonstrated that NADH, molecular oxygen, and the three enzymes, NADH-cytochrome *b*₅ reductase, cytochrome *b*₅, and the terminal desaturase, are involved in the $\Delta 6$ -desaturation reaction (Okayasu et al., 1981). The enzyme $\Delta 5$ -desaturase catalyzes the final step in production of the 20-carbon atom PUFAs arachidonic acid and eicosapentaenoic acid by introducing a *cis*-double bond at the 5, 6 position from the carboxyl end of FAs. This desaturase shares all the conserved characteristics displayed by other front-end desaturases, such as $\Delta 6$ -desaturase (Pereira et al., 2003). The role of $\Delta 9$ -, $\Delta 6$ -, and $\Delta 5$ -desaturase in the synthesis of unsaturated FAs in mammals is shown in Figure 4.

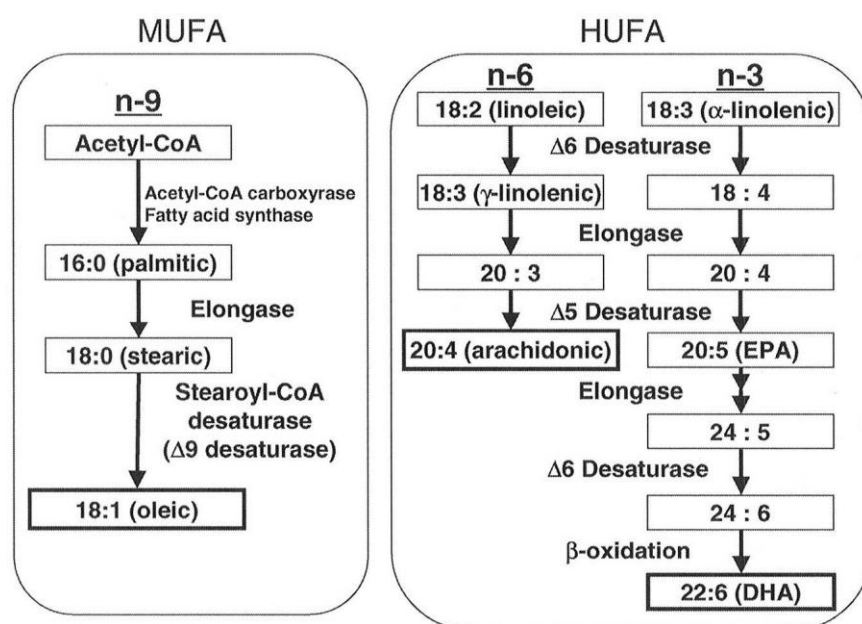


Figure 4 The role of $\Delta 9$ -, $\Delta 6$ -, and $\Delta 5$ -desaturase in synthesis of unsaturated fatty acids in mammals. MUFA, monounsaturated fatty acids; HUFA, highly unsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid (Nakamura and Nara, 2004).

In cows, $\Delta 9$ -desaturase is regulated by dietary factors both at the transcriptional level and the post-transcriptional level (Bernard et al., 2008). The most important factors regulating lipogenic gene expression in the mammary gland are thought to be sterol-response element-binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs). Fatty acids or cholesterol acts by binding to the nuclear receptors PPAR, whereas FAs induce changes in the nuclear abundance of SREBP (Bernard et al., 2008). Coordinated transcriptional regulation of FA desaturases and elongases in mammals are shown in Figure 5. Few studies have investigated the nutritional regulation of $\Delta 9$ -desaturase mRNA abundance and/or protein activity in the ruminant lactating mammary gland. In contrast to other species, nutritional factors have a modest effect on the level of $\Delta 9$ -desaturase mRNA in cows (Bernard et al., 2008). However, the same cited authors reported a decrease in the level of $\Delta 9$ -desaturase mRNA when cows were fed “protected” fish oil.

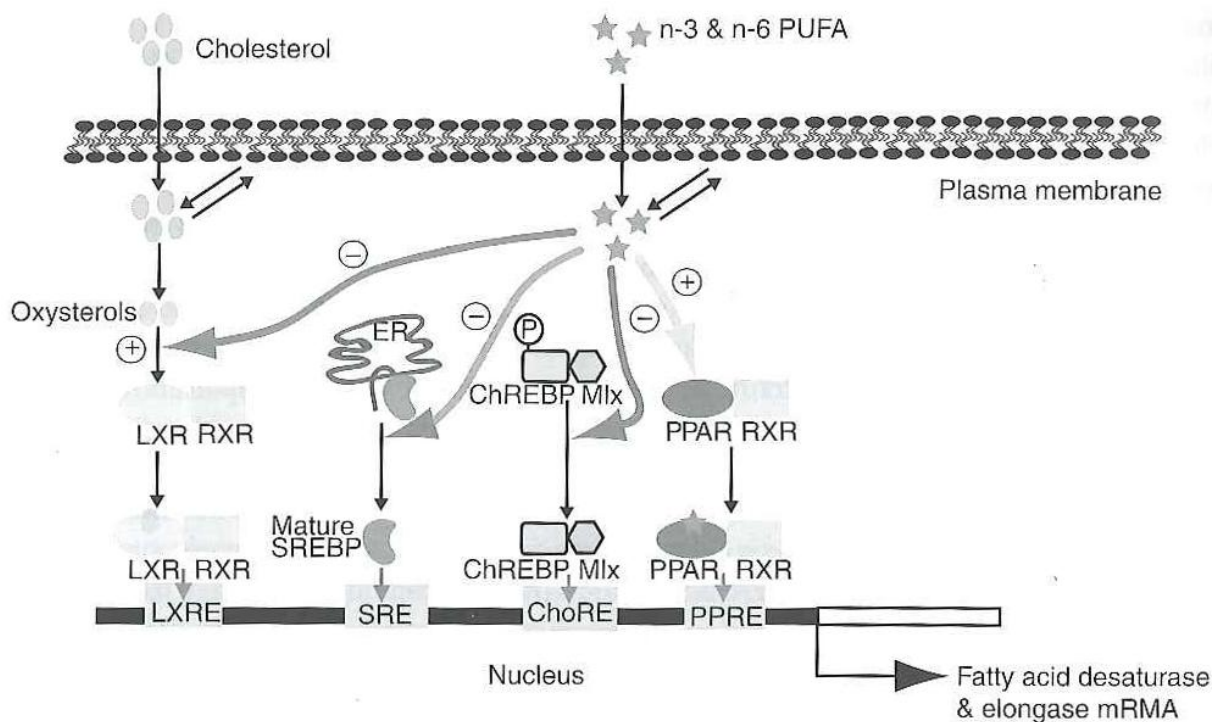


Figure 5 Coordinated transcriptional regulation of fatty acid desaturases and elongases in mammals. PUFA, polyunsaturated fatty acids; +, stimulation; -, inhibition; LXR, liver X receptor, RXR; retinoid X receptor; SREBP, sterol regulatory element binding protein; ChREBP, carbohydrate response element binding protein; Mlx, Max-like receptor; PPAR- α , peroxisome proliferator activated receptor alpha; LXRE, liver X receptor response element; SRE, sterol response element; ChoRE, carbohydrate response element; PPARE, peroxisome proliferator response element (from Miyazaki and Ntambi, 2008).

The regulation of $\Delta 9$ -desaturase has been extensively studied in the liver of rodents, and numerous factors are known to affect regulation of the enzyme (Table 3). In general, the expression of $\Delta 9$ -desaturase is sensitive to several dietary components, such as glucose (Ntambi, 1992, Jones et al., 1998), fructose (Waters and Ntambi, 1994), PUFAs (Ntambi, 1992), cholesterol (Landau et al., 1997, Repa et al., 2000, Kim et al., 2002), vitamin A (Miller et al., 1997, Repa et al., 2000, Samuel et al., 2001, Zolfaghari and Ross, 2003), alcohol (Rao et al., 1984, McCoy et al., 1985), phenolic compounds (Ntambi, 1999), Fe (Kashiwabara et al., 1975, Pigeon et al., 2001) and zinc (Zn) (Sun et al., 2007). Of the minerals, excess Fe increases gene expression (Pigeon et al., 2001), whereas excess Zn decreases gene expression (Sun et al., 2007).

Post-transcriptional activity of $\Delta 9$ -desaturase may also be affected by different nutritional factors. Some studies have reported alterations in activity of the terminal enzyme caused by overload of specific minerals; $\Delta 9$ -desaturase enzyme activity increases with Fe overload (Pigeon et al., 2001) and decreases with cadmium (Cd) overload (Kudo et al., 1991), whereas the effect of copper (Cu) is unclear as it is reported to both increase (Elliot and Bowland, 1968, Ho and Elliot, 1973) and decrease (Sreekrishna and Joshi, 1980) the activity of $\Delta 9$ -desaturase. Deficiencies of minerals can also alter $\Delta 9$ -desaturase activity, and whereas Zn deficiency is reported to increase $\Delta 9$ -desaturase activity (Clejan et al., 1981, Cunnane and Wahle, 1981), deficiency of Fe (Rao et al., 1980, Rao et al., 1983) and Cu (Wahle and Davies, 1974, Wahle and Davies, 1975) is reported to decrease $\Delta 9$ -desaturase activity. Moreover, interactions between Cd and Zn (Kudo et al., 1991), and possibly between Cu and Zn (Cunnane, 1982) could also affect the activity of $\Delta 9$ -desaturase. Accordingly, the regulation of $\Delta 9$ -desaturase by minerals is complex and not well understood.

Table 3 Regulation of $\Delta 9$ -desaturase (Ntambi and Miyazaki, 2003)

Dietary factor	Hormone	Other
↑Phenolic compounds [2]	↑Insulin [17,24]	↑Developmental processes [34,35]
↑Glucose [11,16]	↑Growth hormone [24,25]	↑Temperature [36,37]
↓Polyunsaturated fatty acids [11]	↓Thyroid [26]	↓Thiazolidinediones [38,39]
↑Fructose [17]	↑Androgen [27,28]	↓Cadmium [42]
↑Vitamin A [18,21–23]	↓Leptin [29]	↑Peroxisome proliferators [43,44]
↑Cholesterol [18–20]	↓Glucagon [30]	↑Liver X receptor agonist [45]
↑Iron [40,41]	↑Estrogen [31]	
↑↓Alcohol [46,47]	↑↓Dehydroepiandrosterone [32,33]	

In addition to the minerals listed above, *per os*, intraruminal and intravenous administration of high amounts of Co lowers $\Delta 9$ -desaturation in the mammary gland of lactating dairy cows (Shingfield et al., 2006, Shingfield et al., 2008, Taugbøl et al., 2008, Taugbøl et al., 2010). However, the mechanisms behind this suppression of $\Delta 9$ -desaturation as a result of excess Co are not fully understood. Shingfield et al. (2008) presented one possible working hypothesis, which proposes that Co interferes with the transfer of electrons from cytochrome b5 to the di-Fe protein centre (Lindqvist et al., 1996) of the terminal desaturase. Taugbøl et al. (2010) presented a similar hypothesis, stating that high concentrations of loosely bound Co directly interfere with the insertion of Fe into the $\Delta 9$ -desaturase enzyme, thereby reducing enzymatic activity in mammary cells.

The other desaturases, $\Delta 5$ - and $\Delta 6$ -desaturases, are also influenced by dietary factors. Generally, PUFAs are known to suppress all three desaturases (Holloway and Holloway, 1975, Peluffo et al., 1976, Ntambi et al., 1996, Cho et al., 1999a, Cho et al., 1999b). Deficiency of Zn is also reported to affect the activity of both $\Delta 5$ - and $\Delta 6$ -desaturase (Cunnane and Wahle, 1981, Clejan et al., 1982, Kudo et al., 1990). However, the results are ambiguous, and further investigation is therefore needed to understand the effect of minerals on these enzymes.

2.2.1.4 Triacylglycerol synthesis

Before the FAs are secreted into milk, they are esterified to TAGs. The first step in triacylglycerol biosynthesis is esterification at the sn-1 position by glycerol-3 phosphate acyl transferase (GPAT) (Bernard et al., 2008). Secondly, acyl glycerol phosphate acyl transferase (AGPAT) inserts fatty acyl-CoA at the sn-2 position (Bernard et al., 2008). Acyl glycerol phosphate acyl transferase has a great affinity for saturated fatty acyl-CoA (Mistry and Medrano, 2002) in the order 16:0 > 14:0 > 12:0 > 10:0 > 8:0 (Marshall and Knudsen, 1977).

The final step in the biosynthesis of TAGs is catalysed by diacylglycerol acyltransferase (DGAT) (Yen et al., 2008). Diacylglycerol acyltransferase is the only protein that is specific to triacylglycerol synthesis and may therefore play an important regulatory role (Mayorek et al., 1989). There are two distinct DGAT enzymes, DGAT1 and DGAT2, and although both are involved in TAG synthesis, they have different protein sequences and differ in their biochemical, cellular and physiological functions (Yen et al., 2008). The DGAT enzymes are located in the ER (Stone et al., 2006), where DGAT2 is collocated with $\Delta 9$ -desaturase (Man

et al., 2006). The DGAT enzymes catalyse the formation of TAGs from DAGs and acyl-CoA (Cases et al., 1998) by acylating at the sn-3 position of DAGs (Bionaz and Loor, 2008). Short-chain FAs and *cis*-9 18:1 are reported as preferred substrates for the DGAT enzymes (Palmquist et al., 1993, Ntambi and Miyazaki, 2004). Few studies are performed to investigate DGAT enzymes in the mammary gland of cows, but Bionaz and Loor (2008) reported that the relative mRNA abundance of DGAT1 was 17-fold greater compared to DGAT2 in the mammary gland and that the temporal pattern in expression of DGAT1 was similar to the yield of 4:0. Surprisingly, the results also indicated that DGAT1 was of minor importance in the overall process of milk fat synthesis, but both DGAT1 and DGAT2 were upregulated during lactation (Figure 2)

Compared to ruminants, the DGAT enzymes are investigated to a greater extent in various cell lines and tissues of other species. The activity of DGAT may be primarily determined by substrate availability, and expression of the enzymes is supposed to be regulated at the mRNA level (Yen et al., 2008). However, the transcription factors regulating DGAT genes have not been studied in detail, and there appears to be a reciprocal relationship in the physiological regulation of DGAT1 and DGAT2 (Yen et al., 2008).

2.2.2 Milk fat synthesis in sow vs. ruminants

Pigs are monogastric animals, and accordingly, the substrates absorbed from the digestive tract differ from those in ruminants. This has consequences for lipid metabolism and milk fat synthesis, as highlighted below.

In the sow, direct incorporation of FAs from blood into milk fat is quantitatively less important than in the cow. Accordingly, *de novo* synthesis within the mammary gland is correspondingly more important (Spincer et al., 1969). As sows are simple-stomached animals, FAs absorbed from the digestive tract and taken up by the mammary gland reflect the FAs in the feed. Accordingly, FAs taken up by the mammary gland of sows are more unsaturated compared to the FAs taken up by the mammary gland of cows. Lactating sows may also mobilise body fat to meet their energy requirement (Noblet et al., 1990). However, information about the proportion of mobilised body fat in sow's milk fat is scarce.

In contrast to cow mammary tissue, the mammary tissue of sows synthesise FAs mainly from glucose, and to a lesser extent from acetate (Linzell et al., 1969, Spincer et al., 1969). In

addition, sow mammary tissue is able to synthesise FAs with chain-length up to 18 carbon atoms (Linzell et al., 1969, Spincer et al., 1969). Typical for sows milk is low proportion of short- and medium-chain FAs, indicating that the activity of thioesterase II in sow mammary tissue must be absent, or at least very low. However, the role of thioesterase II in sow mammary tissue has not been elucidated as far as we know.

Little is published on the activity of $\Delta 9$ -desaturase in lactating sows. However, Bickerstaffe and Annison (1970) reported that sows exhibit desaturase activity in mammary tissue similar to that in ruminants.

There are no main fundamental differences between sows and ruminants in the esterification of FAs in the synthesis of TAGs.

3 SUMMARY OF PAPERS I-III

3.1 Paper I

Cobalt reduces the $\Delta 9$ -desaturase index of sow's milk

The objective of this study was to examine if Co reduces $\Delta 9$ -desaturase indices in blood lipids and milk from sows. The experimental design was repeated measurement consisting of a pre-treatment period of a minimum of 9 days, a treatment period of 5 days and a post-treatment period of 6 days. Experimental animals were 10 lactating sows: five sows were injected with 59 mg Co diluted in 5 ml of saline solution, intramuscularly twice a day; and five sows were injected with 5 ml of saline solution, intramuscularly twice a day, as a control. Fatty acid composition and content of Co and Fe in milk was measured on 6 separate days during the experiment, and FA composition of TAG and PL fractions of blood plasma was measured twice during the experiment.

Main results

- Milk $\Delta 9$ -desaturase indices for *cis*-9 18:1, *cis*-9 16:1 and *cis*-9 14:1 were significantly reduced by Co treatment. The effect on desaturase indices appeared already one day after the Co treatment started, and was back to normal 3 days after the treatment ended. The proportion of 20:0 in milk increased with Co treatment.
- Cobalt treatment affected the $\Delta 9$ desaturase indices of neither the triglyceride fraction nor the PL fraction of plasma, but *cis*-11 18:1 was lowered in the PL fraction.
- Cobalt content of milk increased after Co treatment, whereas Fe content was unaffected.
- Sows receiving Co showed decreased feed intake and body weight, but the differences were not statistically significant ($p > 0.05$).

Main conclusion

It is concluded that intravenously injected Co reduces desaturase indices in milk from sows as it does in milk from dairy cows, but the effects are less in sows. Plasma desaturase indices were not affected by Co treatment in sows.

3.2 Paper II

The effect of excess cobalt on milk FA profiles and transcriptional regulation of SCD, FASN, DGAT1 and DGAT2 in the mammary gland of lactating dairy cows

The objective of this study was to investigate the effect of excess Co on gene expression of stearoyl-CoA desaturase (SCD), FASN, DGAT1 and DGAT2 in lactating dairy cows in relation to milk FA profile. Seven multiparous cows of the Norwegian Red Cattle breed (NRF) had their basal diet supplemented with 1.4 g Co as a 24 g/L solution of Co acetate per os twice daily for 7 days, followed by a 9-day depuration period. Udder biopsies were performed prior to the treatment period, after one week of treatment, and immediately after the depuration period. Gene expression was measured using real competitive PCR (rcPCR). Milk yield was recorded daily, chemical composition of milk was measured on 9 separate days during the experiment, and FA composition of milk was measured on the days before the udder biopsies.

Main results

- Excess Co decreased desaturase indices for *cis*-9 18:1, *cis*-9 16:1 and *cis*-9 14:1 in milk. Proportions of all *cis*-9 MUFAs were reduced, and proportion of 18:0 was increased. Milk fat yield was not affected by Co treatment.
- Gene expression levels of SCD, FASN, DGAT1 and DGAT2 in mammary tissue were not significantly affected by Co treatment.
- Milk FA composition was not completely recovered 8 days after the treatment was ended.

Main conclusion

It is concluded that oral administration of excess Co highly reduces desaturase indices in milk fat, and that this effect on desaturase indices is not mediated by altered gene expression level of Δ 9-desaturase, but is exerted at the post-transcriptional level.

3.3 Paper III

Effect of different levels of supplied cobalt on the fatty acid composition of bovine milk

In previous experiments, the administration of high amounts of Co decreased the proportion of MUFAs in bovine milk. The present experiment was conducted to elucidate the amount of Co needed to obtain this effect. Four high-yielding dairy cows, equipped with ruminal cannulae, were used in a 4x4 Latin square. The basal diet consisted of concentrate mixture (9 kg/day) without added Co, and grass silage (*ad libitum*). Four levels of Co were administered as Co-acetate dissolved in distilled water; No Co (T1), 4.0 mg Co/day (T2), 360 mg Co/day (T3), and 5100 mg Co/day (T4). During the treatment periods, the solutions were continuously infused into the rumen. Milk yield and milk content of fat, FAs, protein, lactose, Co, Zn, Fe and Cu were determined. Blood plasma was analysed for content of FA, Co, Zn, Fe and Cu. Feed intake and total tract digestibility of the feed components were also determined.

Main results

- With a few minor exceptions, only the highest level of Co (T4) affected FA composition.
- In milk fat, the proportion of MUFAs was significantly decreased, and the proportion of oleic acid was reduced by as much as 38 % at T4 compared with T1 (12.9 vs. 20.7 g/100 g FA).
- In general, the effects of Co on FA composition of blood were insignificant compared to milk.
- The highest Co level suppressed feed intake and milk yield.

Main conclusion

In conclusion, supplementation with 5100 mg Co/day, significantly reduced the proportion of MUFAs, and increased the proportion of SFAs in milk fat, indicating reduced activity of $\Delta 9$ -desaturase. The amount of Co needed to reduce the proportion of MUFA in bovine milk fat, is probably between 20 and 50 mg Co/kg diet DM, which is on the order of 100-200 times the

normal Co intake of lactating dairy cows. Thus, Co intake within the normal variation in practise would have no significant effect on milk FA composition.

4 GENERAL DISCUSSION

The enzyme $\Delta 9$ -desaturase catalyses the synthesis of MUFAs, and is a key regulator of fat metabolism. Suppression of its activity by supplying excess amounts of Co significantly affects bovine milk FA composition (Shingfield et al., 2006, Shingfield et al., 2008, Taugbøl et al., 2008, Taugbøl et al., 2010), and thereby negatively affects the nutritional quality of milk for human consumption. Moreover, the alteration in the ratio between MUFAs and SFAs affects membrane PL composition, and change in this ratio has been implicated in a variety of disease states (Ntambi and Miyazaki, 2004). Accordingly, the finding that excess Co lowers $\Delta 9$ -desaturase activity is of great interest, and justifies further research to elucidate different aspects of Co in relation to desaturation of MUFAs. The results also strongly indicate that excess Co affects the activity of $\Delta 6$ -desaturase and maybe $\Delta 5$ -desaturase as well (Taugbøl et al., 2010, Paper II, Paper III). These desaturases play a central role in the desaturation of *cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3, and their product FAs have essential functions in fat metabolism and influence fat characteristics (Nakamura and Nara, 2004). Because of the central role of PUFAs in the body, alteration in the activity of $\Delta 5$ - and $\Delta 6$ -desaturases is also of great interest. The most important aspects of our findings are discussed more in detail below.

4.1 Delta 9-desaturase activity in sows vs. dairy cows

Sow mammary tissue is effective in converting 18:0 into *cis*-9 18:1 (Bickerstaffe and Annison, 1970). However, our results (Paper I) clearly show a lesser effect of intramuscular injections of excess Co on the proportion of MUFA in sow's milk compared to the effects on cow's milk obtained with *per os*, intravenous or intraruminal administration of excess Co (Taugbøl et al., 2008, Taugbøl et al., 2010, Paper II, Paper III). The most pronounced difference in response between sows and dairy cows was for 18:1. In sow's milk, the $\Delta 9$ -desaturase index for 18:1 was reduced by only 3-4% as opposed to 42% in cow's milk. There was also a lesser effect of excess Co on $\Delta 9$ -desaturase indices for 14:1 and 16:1 in sow's milk compared to cow's milk, but the differences were relatively small: 42 vs 67 % and 38 vs 67 %, respectively (Paper I and III). These differences in response between sows and cows are expected, and support the view that the main regulatory factor of $\Delta 9$ -desaturase is the supply of PUFAs, which inhibit $\Delta 9$ -desaturation (Ntambi, 1992, Ntambi et al., 1996, Sessler et al.,

1996, Ntambi, 1999). In most feeds for ruminants, as well as for sows, 18:0 constitutes only a small part compared to unsaturated 18-carbon FAs (Harstad and Steinshamn, 2010). Due to the extensive biohydrogenation of feed unsaturated FAs in the rumen of cows (Doreau and Ferlay, 1994, Looor et al., 2004, Palmquist, 2006, Jenkins and Bridges, 2007), the supply of 18:1 (and PUFAs) to the mammary gland of cows is insignificant compared to 18:0. Thus, the need for desaturation is high in the mammary gland of cows, and the supply of the main substrate (18:0) is abundant even though the mammary gland of cows is not able to synthesise 18:0 (Annison et al., 1967, Bines and Brown, 1968). In contrast, there is an abundant supply of unsaturated FAs to the mammary gland of sows, whereas the supply of 18:0 (substrate) from the feed is negligible. Accordingly, the need for desaturation is not so critical in the sow udder, and in addition to the inhibitory effect of PUFAs on $\Delta 9$ -desaturase, the supply of substrate to the mammary gland may be a limiting factor. However, the extent of *de novo* synthesis of 18:0 may be significant (Linzell et al., 1969, Spincer et al., 1969). Compared to 18-carbon FAs, the situation is completely different, especially for FA with 14-carbon atoms, but also for FAs with 16 carbon atoms. In most commonly used feeds, the proportions of 14:0 and 14:1 are usually low (<1% of the FAs) (Harstad and Steinshamn, 2010). Thus, these FAs in milk from both sows and cows, originate almost quantitatively from *de novo* synthesis in the mammary gland. Accordingly, the FAs 14:0 and *cis*-9 14:1 give the best estimation for the response of mammary $\Delta 9$ -desaturase activity (Bernard et al., 2008). The proportion of 16:0 in the fat fraction of feeds differs, whereas the proportion of 16:1 is insignificant in most feeds (Harstad and Steinshamn, 2010). Thus, as relatively insignificant amounts of *cis*-9 14:1 and *cis*-9 16:1 originate from the feed, the degree of desaturation of 14:0 and 16:0 in the mammary gland largely depends on the need for desaturating FAs. The desaturation of FAs was apparently more critical in cow's mammary gland (Paper I to III).

4.2 Effect of physiological status on $\Delta 9$ -desaturase activity

Contrary to milk FA composition, the effects of excess Co on plasma FA composition were small and relatively insignificant in both sows and dairy cows (Paper I and III). However, excess Co slightly decreased proportions of MUFAs in blood plasma when administered to dairy cows (Paper III), indicating that the effect of Co is general and not limited to the lactating mammary gland. $\Delta 9$ -Desaturase activity in the liver of ruminants and pigs is normally low, and adipose tissue is the primary site of $\Delta 9$ -desaturase activity in non-lactating

animals (Chang et al., 1992, Kouba et al., 1997). However, at the onset of lactation, expression of $\Delta 9$ -desaturase in adipose tissue of ruminants is largely downregulated, whereas expression of $\Delta 9$ -desaturase in the mammary gland is considerably upregulated (Ward et al., 1998, Bionaz and Loor, 2008). In sows, adipose tissue lipid metabolism is unaffected by lactation if sufficient energy is available (Parmley et al., 1996). However, in situations of negative energy balance, as was probably the case in the present experiment (Paper I), adipose tissue lipid anabolism can be dramatically reduced (Prentice and Prentice, 1988, Mcnamara, 1995, Parmley et al., 1996). Accordingly, the absent or low activity of $\Delta 9$ -desaturase in the liver and adipose tissue of both lactating sows and in lactating dairy cows, could explain the relatively insignificant effect of excess Co on the composition of blood plasma FAs.

4.3 Mechanisms behind alterations in fatty acid compositions by excess cobalt

Desaturation

The regulation of $\Delta 9$ -desaturation in the mammary gland can be altered either by affecting the level of gene expression of the enzyme or by affecting the activity of the enzyme post-transcriptionally or both. The mRNA abundance of $\Delta 9$ -desaturase in the mammary gland of lactating dairy cows is large relative to other classical lipogenic enzymes. Accordingly, $\Delta 9$ -desaturase probably plays a crucial role in TAG synthesis (Bionaz and Loor, 2008).

Compared to PUFAs, which are the best known regulators of $\Delta 9$ -desaturase gene expression [reviewed by Ntambi (1999)], information about the effect of minerals on $\Delta 9$ -desaturase in the lactating mammary gland is scarce. However, both Fe and Zn (Pigeon et al., 2001, Ntambi and Miyazaki, 2004, Sun et al., 2007) have the ability to regulate $\Delta 9$ -desaturase gene expression in various tissues and species. On the contrary, the results of our study showed that excess Co had no significant effect on gene expression levels of the $\Delta 9$ -desaturase enzyme (Paper II). We therefore postulated that the effect of Co on milk FA profile was probably at the post-transcriptional level by reducing the activity of $\Delta 9$ -desaturase in the mammary gland. Another mineral shown to decrease the enzyme activity of $\Delta 9$ -desaturase post-transcriptionally when administered in excess is cadmium (Kudo et al., 1991). On the other hand, excess Fe increases $\Delta 9$ -desaturase activity (Pigeon et al., 2001). The mechanism behind these changes is not known. Factors regulating the enzyme activity of $\Delta 9$ -desaturase post-transcriptionally seem to be poorly investigated.

Both $\Delta 5$ - and $\Delta 6$ -desaturase share common regulatory mechanisms of gene expression with $\Delta 9$ -desaturases, such as the transcription factors SREBP-1c, PPAR- α , liver X receptor (LXR) and carbohydrate response element binding protein (ChREBP) (Miyazaki and Ntambi, 2008). Therefore, as gene expression levels of $\Delta 9$ -desaturase are probably unaffected by excess Co (Paper II), gene expression levels of $\Delta 5$ - and $\Delta 6$ -desaturase may also be unaffected. However, contrary to $\Delta 9$ -desaturase, the relative abundance of $\Delta 5$ - and $\Delta 6$ -desaturase in the mammary gland of dairy cows is low (Bionaz and Looor, 2008). This resulted in unreliable estimates for $\Delta 6$ -desaturase gene expression levels in our study (Paper II), and no conclusion could be drawn regarding the effect of excess Co on this gene (Paper II).

Different mechanisms may explain a post-transcriptional regulation of desaturases, and there may be a common mechanism affecting both $\Delta 6$ - and $\Delta 9$ -desaturases. Even though both $\Delta 5$ - and $\Delta 6$ -desaturases differ from $\Delta 9$ -desaturase by containing a fused cytochrome b5 domain at their N-terminus (Cho et al., 1999b, Cho et al., 1999a, Leonard et al., 2000, Sperling and Heinz, 2001), microsomal cytochrome b5 also plays an important role in the process of $\Delta 6$ -desaturation (Okayasu et al., 1981, Guillou et al., 2004). A potential role of microsomal cytochrome b5 in the process of $\Delta 5$ -desaturation has, to our knowledge, not been investigated. Accordingly, microsomal cytochrome b5 may possibly represent dissimilarity between $\Delta 5$ -desaturase and the other two desaturases, explaining the divergent effect of excess Co on this desaturase.

Cytochrome b5 is a haemoprotein (Schenkman and Jansson, 2003), and one possible hypothesis, with the potential to influence both $\Delta 6$ - and $\Delta 9$ -desaturation, is therefore that Co induces heme oxygenase (Maines and Kappas, 1974, Maines and Kappas, 1975, Maines and Kappas, 1976b, Maines and Kappas, 1976a) and subsequently causes a degradation of the haem group of cytochrome b5 and inhibits the transfer of electrons from cytochrome b5 to the di-Fe protein centre of $\Delta 9$ -desaturase (in line with the hypothesis of Shingfield et al (2008)). The transfer of electrons from cytochrome b5 to the terminal $\Delta 9$ -desaturase is central in the oxidation-reduction reaction required for the conversion of acyl-CoA substrates to MUFAs (Dailey and Strittmatter, 1980, Mitchell and Martin, 1995). In study 3 (Paper III), an increase in Fe concentration in blood plasma, and a significant increase in milk, may strengthen the hypothesis of induced haem oxygenase. However, an effect of Co by directly interfering with the insertion of Fe into the terminal enzymes, as suggested by Taugbøl et al. (2010), is also possible. Therefore, no conclusion on mechanisms can be drawn based on the present

experiments, and functional studies are required to understand the molecular interactions mediated by Co in the FA desaturation process.

Fatty acid and triacylglycerol synthesis

Excess Co affected the proportion of some individual *de novo* synthesised FA, as 4:0, 8:0 and 12:0 (Paper II). These changes are probably not related to lower action of the desaturases. It is known that the enzyme FASN has a major role in *de novo* synthesis of FAs (Wakil, 1989), but the exact mechanism determining the chain length of *de novo* synthesized FAs is not known.

However, excess Co had no effect on gene expression levels of FASN. (Paper II).

Accordingly, excess Co probably affects the action of FASN through post-transcriptional mechanisms.

The enzymes DGAT1 and DGAT2 are responsible for the synthesis of TAGs from DAGs and acyl-CoA (Cases et al., 1998). They acylate the *sn*-3 position of DAG, and most of the 4:0 in milk TAG is found in this position (Jensen, 2002). DGAT2 is collocated with Δ 9-desaturase in the ER, and it is suggested that the FAs 16:0 and 18:0 are desaturated by Δ 9-desaturase and then channelled to DGAT2 for the final step in TAG synthesis (Man et al., 2006). The regulation of this process is poorly investigated, but both expressional and post-transcriptional regulation is suggested (Coleman and Lee, 2004). In the study by Taugbøl et al. (2010), as well as in our experiments (Paper II and III), the proportion of 4:0 in milk fat increased, whereas the proportion of *cis*-9 18:1 decreased. Bionaz and Looor (2008) found that gene expression of DGAT1 was similar to the yield of 4:0. However, despite the increased proportion of 4:0 in milk fat and unaffected fat yield, gene expression level of DGAT1 was not affected (paper). On the other hand, the results showed that gene expression levels of DGAT2 tended to be upregulated as a result of excess Co (Paper II). Short-chain FA esterificated at the *sn*-3 position of glycerol decreases the melting point of milk fat (Palmquist et al., 1993). Therefore, one possible hypothesis is that this is a mechanism to compensate for the higher amount of SFAs. However, the effect on DGAT2 was not significant, and more results are needed to confirm the effects on this gene and the rationality behind the potential upregulation.

4.4 Effect of cobalt level on $\Delta 9$ -desaturase activity

Cobalt is essential as a trace mineral in the diet of ruminants, and the requirement is set at 0.11 mg/kg diet DM (NRC, 2001). Due to local occurrence of soils deficient in Co, the feeds may not meet the animal requirement (Ammerman and Goodrich, 1983). Therefore, to ensure adequate intake, small amounts of Co (<1 mg/kg DM) are normally added to the concentrate mixtures of dairy cows. Additionally, cows may receive extra Co-containing mineral supplements. Accordingly, dairy cows probably receive Co in excess of their requirement under certain feeding conditions. The reduction in proportion of MUFAs in milk fat as a result of excess Co obtained in earlier experiments were considerable (Shingfield et al., 2006, Shingfield et al., 2008, Taugbøl et al., 2008, Taugbøl et al., 2010), and it was therefore of interest to examine if lower levels of Co also decrease the proportions of MUFAs in milk fat.

Our results demonstrated that very high amounts of Co (>17 mg/kg diet DM) were needed to affect the milk FA composition (Paper III), and only the highest level of Co (270 mg/kg diet DM) significantly altered milk FA composition. However, similar results were obtained with lower levels of Co in our second study, where the proportion of MUFAs was significantly reduced by administering Co at 150 mg/kg diet DM (Paper II). An even lower level (51 mg/kg diet DM) significantly reduced the proportion of MUFAs in milk fat in a study by Shingfield et al. (2008). Accordingly, the amount of Co needed to significantly reduce the proportion of MUFAs in bovine milk fat is probably in the range of 20-50 mg/kg diet DM. Based on the present results, there is therefore no risk that the levels of Co normally supplied in dairy cows significantly affect milk FA composition. Anyway, the level of Co needed for affecting bovine milk proportion of MUFA is high.

The toxic level of Co for ruminants is set at 25 mg/kg feed DM (NRC, 2005). However, despite the Co levels far above 25 mg/kg feed DM in the present experiments (Paper II and Paper III), no obvious signs of toxicity were observed, except for reduced feed intake and milk yield, reported with the highest level of Co in the last experiment (Paper III). Still, it has to be kept in mind that both studies were short-term, with the treatment periods lasting a maximum of 11 days. Therefore, no conclusions can be drawn from the present experiments with regard to the long-term effects of such high amounts of Co.

MAIN CONCLUSIONS

1. High Co levels significantly lower the proportion of MUFAs in milk fat from lactating sows. This result confirms that excess Co suppresses $\Delta 9$ -desaturase activity in lactating sows and, therefore, probably also in other species, including humans. The activity of $\Delta 9$ -desaturase in the udder of lactating sows is lower compared to dairy cows. This is probably because the supply of MUFA to the udder is higher in sows than in dairy cows, and hence the need for desaturation of MUFA is less in sows.
2. Administration of excess Co *per os* to lactating dairy cows dramatically reduced the proportion of MUFAs in milk fat, but did not affect gene expression levels of $\Delta 9$ -desaturase in the mammary gland. Accordingly, the effect of excess Co on $\Delta 9$ -desaturation is mediated at the post-transcriptional level. The exact mechanism behind the inhibition of the $\Delta 9$ -desaturase enzyme is, however, not known.
3. High amounts of Co are needed to reduce $\Delta 9$ -desaturation in dairy cows. The exact amount is not known, but is probably in the range of 20-50 mg/kg DM. Accordingly, the normal level of Co in the diet of dairy cows will not significantly affect the milk fat proportion of MUFAs.
4. Excess Co slightly reduced plasma content of MUFAs in dairy cows, but the effects were less compared to milk. Therefore, excess Co probably has a general effect on $\Delta 9$ -desaturase, and not limited to the mammary gland.
5. The results of the present experiments strongly indicate that excess Co also suppresses the activity of $\Delta 6$ -desaturase, but not $\Delta 5$ -desaturase. However, this has not been confirmed.

IMPLICATIONS AND PERSPECTIVE

Cobalt–EDTA has been extensively used as a liquid phase digestion marker in digestibility and passage studies in ruminants. However, due to its effect on $\Delta 9$ desaturase, it is not recommended to use Co-EDTA as a digestion marker in experiments with ruminants involving parameters of fat metabolism. In pigs, Co-EDTA is also used as a digestion marker. However, the rate of absorption of Co from Co-EDTA is, to our knowledge, not known in pigs. Therefore, the absorption of Co-EDTA, and the potential effect on $\Delta 9$ -desaturation should be elucidated to decide whether or not Co-EDTA is a suitable liquid phase marker for digestibility experiments in pigs.

The amount of Co required to lower $\Delta 9$ -desaturation is much higher than the intake of Co of dairy cows in practise. Thus, from that point of view, there is no reason to change the amounts of Co recommended.

Future research should focus on elucidating the mechanisms behind the post-transcriptional suppression of $\Delta 9$ -desaturase. By understanding these mechanisms, it may be possible to obtain the opposite effect, and thereby increase the nutritional quality of milk for human consumption. Moreover, because $\Delta 5$ - and $\Delta 6$ -desaturases play key roles in fat metabolism, future research should also focus on elucidating their regulatory mechanisms.

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Paper I

Cobalt reduces the Δ^9 -desaturase index of sow milk



ORIGINAL ARTICLE

Cobalt reduces the Δ^9 -desaturase index of sow milkI. J. Karlengen¹, O. M. Harstad¹, N. P. Kjos¹, B. Salbu², A. H. Aastveit³ and O. Taugbøl¹¹ Department of Animal and Aquacultural Sciences, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway,² Department of Plant and Environmental Sciences, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway, and³ Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway**Keywords**sow milk, cobalt, Δ^9 -desaturase, oleic acid, palmitoleic acid**Correspondence**

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The main objective of this study was to examine if cobalt (Co) reduces Δ^9 -desaturase indices in milk and blood lipids in sows. The experimental design consisted of a repeated measurement consisting of a pre-treatment period of a minimum of 9 days, a treatment period of 5 days and a post-treatment period of 6 days. Experimental animals consisted of 10 lactating sows; five sows had an intramuscular injection of 59 mg Co diluted in 5 ml 0.9% saline solution twice a day, whereas the other five had an *intramuscular* injection of 5 ml 0.9% saline solution twice a day as a control. Milk Δ^9 -desaturase indices for *cis*-9 18:1, *cis*-9 16:1 and *cis*-9 14:1 were significantly reduced ($p < 0.0001$) as a result of the Co-treatment, contrasting with the plasma Δ^9 -desaturase indices, which were unaffected. Potential mechanisms explaining how Co might reduce milk Δ^9 -desaturase indices are discussed. Moreover, the toxicological level of Co and potential implications of using Co-ethylenediaminetetraacetic acid as a liquid marker in digestibility experiments are addressed.

Introduction

Delta-9 desaturase, also referred to as stearyl-CoA desaturase is the rate-limiting enzyme catalyzing the synthesis of monounsaturated fatty acids from saturated fatty acids (Nakamura and Nara, 2004; Paton and Ntambi, 2009). Preferred substrates are 16:0 and 18:0, which are converted into *cis*-9 16:1 and *cis*-9 18:1 respectively (Ntambi, 1995). These fatty acids are major substrates for the synthesis of various types of lipids, such as phospholipids (PL), triglycerides (TG) and cholesterol esters. Thus, Δ^9 -desaturase is important in lipid metabolism, and it shares common features in animals including insects, nematodes and vertebrates (Nakamura and Nara, 2004). It has recently been revealed that Cobalt (Co) reduces the Δ^9 -desaturase indices in bovine milk when supplied in large amounts (Shingfield et al., 2008; Taugbøl et al., 2008; Taugbøl et al., 2010), probably

by influencing the activity of Δ^9 -desaturase (Taugbøl et al., 2010). The Δ^9 -desaturase activity in the bovine mammary gland is high (Kinsella, 1970; Barber et al., 1997; Glasser et al., 2007) because the supply of unsaturated fatty acids to the udder of ruminants is low, this being caused by the extensive biohydrogenation in the rumen of unsaturated fatty acids from the feed (Kinsella, 1970). In monogastric animals, the activity of Δ^9 -desaturase is expected to be lower because of a greater supply of unsaturated fatty acids from the intestine (Kinsella, 1970). However, Spincer et al. (1969) reported that direct incorporation of fatty acids of the plasma TG into milk fat was less important in the sow than in ruminants, their results indicating a substantial *de novo* synthesis of milk fatty acids in sows. Hence, the activity of Δ^9 -desaturase may also be considerable in the udder of monogastrics. To our knowledge, there is no information about the effect of Co on the Δ^9 -desaturase in

monogastrics. The main objective of this study was therefore to examine if Co affects Δ^9 -desaturase indices in milk and blood lipids of lactating sows.

Materials and methods

Experimental design

The experimental design consisted of a repeated measurement of two treatments with a pre-treatment period of at least 9 days, a treatment period of 5 days and a post-treatment period of 6 days.

Animals and treatment

The experimental animals were 10 (three primiparous and seven multiparous) lactating sows (BW of 239 ± 46 kg), all giving birth within a period of eight days. The litter size was 11.7 ± 1.8 piglets. The sows were divided into five groups each with two animals, based on litter number and expected time of farrowing. The two animals within each group were then randomly split to a Co-treated group (Co-group) and a control-group. The five animals in the Co-group had an intramuscular injection of 59 mg Co at 08:00 and 16:00 hours, in the form of 250 mg Co-acetate (Honeywell Speciality Chemicals, Seelze, Germany) dissolved in 5 ml 0.9% saline solution. The daily amount of Co supplied was (on average) 1.9 mg per kg metabolic weight (BW^{0.75}) of the sows. This equalled the amount used in a former experiment with dairy cows (Taugbøl et al., 2010). The five animals in the control-group had an intramuscular injection of 5 ml 0.9% saline solution at 08:00 and 16:00 hours. The sows were in their 7–14th day of lactation when the treatment period started and were housed in pens with partially slotted floors with sawdust provided as bedding. All sows were offered the same concentrate (Table 1) *ad libitum*, fed individually by use of automatic feeders from 3 days before expected farrowing and throughout the whole experiment. The sows had free access to water. Piglets had no additional feeding until after the third week of lactation. Care and handling of the sows conformed to the laws and regulations controlling experiments with animals in Norway (The Animal Protection Act of December 20, 1974, and the Animal Protection Ordinance Concerning Experiments in Animals of January 15, 1996).

Milk sampling and analyzing

Individual milk samples were obtained at 08:00 hours (immediately before intramuscular injections), by hand-milking, 4 and 3 days before the treatment

Table 1 Composition of sow diet (g/kg diet)

Ingredients	
Barley	390
Wheat	350
Soya bean meal, extruded	130
Vegetable fat	30
Fish meal	20
Sugar cane molasses	20
Fixing agent	17
Ground limestone	10.9
Mono calcium phosphate	10.3
Acids	7.1
Sodium chloride	4.3
Amino acids	0.8
Taste premix	0.22
Vitamin premix*	2.96
Mineral premix†	1.6
Enzymes	0.15
Calculated contents	
Crude protein	163
Crude fat	47
Crude fibre	31
Ash	53
Lysine	8.2

*Containing per kg feed: vitamin A, 9900 IU; vitamin D₃, 1080 IU; vitamin E, 165 mg.

†Containing per kg feed: copper(II)sulphate, 16 mg; sodium selenite, 0.37 mg; Sangrovit® (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany), 30 mg; 6-phytase (EC 3.1.3.26), 750 FYT.

period started, days 1, 2, 3 and 5 in the treatment period, as well as days 3 and 6 after the treatment had ended. Oxytocin (0.5–1.0 ml) was injected intramuscularly 5–10 min before sampling to induce the milk-ejection reflex. Milk samples were kept frozen (–20 °C) for later analysis of the fatty acid composition (all samples) and for analysis of the content of Iron (Fe) and Co in the samples withdrawn 4 days before treatment period started and day 5 of the experimental period.

Milk lipids were extracted with ethanol, diethyl ether and petroleum ether (International IDF standard nr 1D: Milk Determination of Fat Content, Reference Method). The extracted lipids were methylated according to Kramer et al. (1997) with NaOCH₃ and HCl/methanol. Fatty acid methyl esters were separated and quantified according to (Taugbøl et al., 2008). Samples of 2 ml milk were analysed for content of Co and Fe by the ICP-OES method according to (Taugbøl et al., 2010).

Blood sampling and analyzing

Blood samples (10 ml heparin-containing tubes) were taken from the jugular vein immediately after

milk samples at 08:00 hours, before intramuscular injections, 3 days before the treatment period started and at day 5 in the treatment period. During blood sampling, sows were fixed using a snout break. Blood was centrifuged at 500 *g* for 20 min and plasma was stored at -20°C for later analysis of fatty acid composition of TG and PL.

Plasma lipids were extracted by adding 0.6 ml 0.5 M KH_2PO_4 , 3 ml chloroform and 1 ml methanol to 0.4 ml plasma. After vortexing for 2 min and centrifugation, the lower phase was collected with a Pasteur pipette through the protein disk and evaporated with hot nitrogen. Lipid classes were separated by Silica TLC eluted with hexane-diethyl ether-formic acid (80/20/2 v/v). Spots were visualised with iodine vapour and the area representing TG and PL were scraped into separate vials. Lipids were extracted from the silica powder with dichloromethane and evaporated by hot nitrogen. Triglycerides were added 1 ml toluene. Then, TG and PL were analysed for fatty acid composition as described for fatty acid profiles in plasma by Taugbøl *et al.* (2008).

Measurements

The individual automatic feeders were refilled every fourth day and the daily feed intake was calculated after subtracting the concentrate left from that supplied. The mean feed intake for day 2 to day 5 in the experimental period was used for statistical analysis. All sows were weighed on day 1 of the experimental period and on day 3 after the treatment was ended. Piglets were weighed immediately after birth, on day 5 in the experimental period and at the time of weaning.

Calculation of Δ^9 -desaturase indices

The Δ^9 -desaturase indices were calculated as: [product of Δ^9 -desaturase]/[product of Δ^9 -desaturase + substrate of Δ^9 -desaturase] (Kelsey *et al.*, 2003). The desaturase index was calculated for three pairs of fatty acids: *cis*-9 14:1-14:0, *cis*-9 16:1-16:0, *cis*-9 18:1-18:0.

Statistical analysis

The GLM procedure of SAS software version 9.1 (SAS Institute, Cary, NC, USA) was applied for the statistical analyses of the data. The model used to test for the effects of the treatment on the fatty acid compositions in milk and plasma, Δ^9 -desaturase indices and Co and Fe in milk was $Y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \gamma_k +$

$\beta\gamma_{jk} + \varepsilon_{ijk}$ where Y_{ijk} is the *ijk*th observation, μ is the overall mean effect, α_i is the *i*th fixed treatment effect, $\beta_{j(i)}$ is the random effect of the *j*th sow within the *i*th treatment, γ_k is the fixed *k*th day effect when the measurement was taken, $(\beta\gamma)_{jk}$ is the fixed interaction effect between treatment and day and ε_{ijk} is the random error.

The effect of treatment on feed intake and weight during the treatment period was analysed according to the model $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$ where Y_{ij} is the *ij*th observation, μ is the general mean, α_i is the effect of treatment and ε_{ij} is the experimental error.

Results

Blood plasma fatty acid composition

The fatty acid composition of plasma TG and PL is presented in Table 2. The Co-treatment had no effect on fatty acid composition of the TG fraction of plasma, whereas in the PL fraction of plasma, only the proportion of *cis*-11 18:1 was significantly lowered ($p = 0.032$) (Table 2), the reduction while small was consistent for all five animals. Co-treatment had no effect on desaturase indices in TG or PL fractions (data not shown).

Milk fatty acid composition and desaturase indices

Milk fatty acid composition and desaturase indices are presented in Table 3 and Fig. 1 respectively. Desaturase indices for *cis*-9 18:1, *cis*-9 16:1 and even *cis*-9 14:1, were significantly reduced ($p < 0.0001$) by the Co-treatment (Fig. 1). This was due either to an increasing effect of Co on the proportion of substrate (18:0) or a lower proportion of the products (*cis*-9 14:1 and *cis*-9 16:1) of Δ^9 -desaturase. The sum of 14:0, 16:0 and 18:0 was approximately 5% units higher and the sum of their corresponding monounsaturated acids (*cis*-9 14:1, *cis*-9 16:1 and *cis*-9 18:1) approximately 5% units lower in the Co-treatment group, in relation to the control group (Table 3). It is noteworthy that the response of desaturase indices appeared already one day after the Co-treatment started ($p < 0.01$) but was back to normal 3 days after the treatment ended (Fig. 1). With exception of arachidic acid (20:0), there were no other consistent differences in milk fatty acid composition between the Co-treatment and control groups (Table 3).

Content of Co and Fe in milk

The Co content in milk was under the detection limit ($< 0.02 \mu\text{g/g}$) for all samples from the pre-treatment

Table 2 Proportions of plasma fatty acids (g/100 g fatty acids) in phospholipids and triglycerides before (pre-treatment period) and after 5 days (treatment period) of intramuscular injections of cobalt (Co-treatment) or saline solution (Control) to lactating sows. Values represent means from five sows

Sampling day Treatment	Phospholipids						Triglycerides					
	Pre-treatment period [†]			Treatment period			Pre-treatment period [†]			Treatment period		
	Control	Co-treatment	p-value [‡]	Control	Co-treatment	SEM	Control	Co-treatment	p-value [‡]	Control	Co-treatment	SEM
Fatty acids												
14:0	0.42	0.39	0.874	0.33	0.27	0.082	1.88	1.89	0.755	1.34	1.66	0.466
<i>cis</i> -9 14:1	0.07	0.04	0.337	0.04	0.03	0.010	0.28	0.23	0.739	0.41	0.40	0.058
15:0	0.22	0.21	0.784	0.16	0.14	0.024	0.42	0.45	0.813	0.29	0.29	0.083
<i>cis</i> -9 15:1	0.25	0.24	0.983	0.32	0.31	0.029	0.31	0.26	0.555	0.70	0.77	0.096
16:0	29.71	26.41	0.577	23.72	22.94	2.175	29.85	30.16	0.706	31.24	34.45	3.717
<i>cis</i> -9 16:1	0.51	0.45	0.681	0.39	0.39	0.069	0.72	0.98	0.351	0.55	0.53	0.142
17:0	0.75	0.76	0.557	0.54	0.61	0.058	0.54	0.82	0.242	0.35	0.37	0.103
<i>cis</i> -9 17:1	0.17	0.14	0.425	0.16	0.17	0.026	0.33	0.26	0.891	0.17	0.12	0.086
18:0	20.89	21.74	0.238	17.26	19.73	0.637	9.25	10.34	0.546	8.72	8.23	1.243
<i>trans</i> -9 18:1	0.33	0.32	0.640	0.27	0.28	0.034	0.66	0.65	0.594	0.60	0.78	0.183
<i>cis</i> -9 18:1	12.38	12.61	0.942	11.48	11.60	0.639	26.48	27.64	0.678	24.50	21.32	5.044
<i>cis</i> -11 18:1	1.67	1.87	0.032	1.64	1.52*	0.061	2.11	2.36	0.322	2.46	1.84	0.409
<i>cis</i> -9, <i>cis</i> -12 18:2	18.11	17.20	0.682	21.28	19.16	1.435	14.96	12.29	0.588	12.53	12.02	1.918
<i>cis</i> -(9,12,15) 18:3	0.40	0.39	0.828	0.63	0.57	0.094	0.92	0.76	0.727	0.92	0.93	0.238
20:0	0.37	0.37	0.133	0.39	0.49	0.029	-	-	-	-	-	-
<i>cis</i> -11 20:1	0.23	0.22	0.995	0.22	0.21	0.023	0.95	0.81	0.913	0.48	0.37	0.168
<i>cis</i> -(8,11,14) 20:3	0.38	0.45	0.286	0.40	0.60	0.056	-	-	-	-	-	-
<i>cis</i> -(5,8,11,14) 20:4	5.01	6.32	0.373	7.13	7.04	0.739	-	-	-	-	-	-
<i>cis</i> -(8,11,14,17) 20:4	0.13	0.13	0.292	0.17	0.22	0.026	-	-	-	-	-	-
22:0	0.23	0.25	0.468	0.26	0.32	0.029	-	-	-	-	-	-
<i>cis</i> -(5,8,11,14,17) 20:5	0.78	1.01	0.432	1.89	1.73	0.239	-	-	-	-	-	-
<i>cis</i> -13 22:1	1.36	1.85	0.821	1.33	1.61	0.425	-	-	-	-	-	-
24:0	0.37	0.40	0.904	0.77	0.75	0.198	-	-	-	-	-	-
<i>cis</i> -(7,10,13,16,19) 22:5	1.93	2.23	0.962	2.42	2.69	0.340	-	-	-	-	-	-
<i>cis</i> -(4,7,10,13,16,19) 22:6	1.23	1.42	0.968	1.59	1.80	0.249	-	-	-	-	-	-

*p < 0.01, in comparison with pre-treatment period within treatment.

†Samples taken 3 days before treatment period started.

‡p-values reported are for testing the interactions between treatments and time.

Table 3 Proportions of milk fatty acids (g/100 g fatty acids) in the treatment period (days 1, 2, 3 and 5) and in the post-treatment period (3 and 6 days after the treatment period was ended) for sows having intramuscular injections of cobalt (Co-treatment) or saline solution (Control). Values represent means from five sows

Sampling day	Treatment period						Post-treatment period						p-value‡	SEM	
	1†		2		3		3		5		6				
	Control	Co-treatment	Control	Co-treatment	Control	Co-treatment	Control	Co-treatment	Control	Co-treatment	Control	Co-treatment			
Fatty acids															
4:0	0.58	0.63	0.58	0.64	0.28	0.38	0.34	0.41	0.15	0.17	0.14	0.15	0.967	0.057	
6:0	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.05	0.04	0.05	0.04	0.05	0.592	0.004	
8:0	0.06	0.06	0.06	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.06	0.05	0.930	0.004	
10:0	0.39	0.34	0.36	0.34	0.37	0.33	0.37	0.29	0.32	0.29	0.40	0.29	0.697	0.025	
12:0	0.36	0.32	0.36	0.34	0.39	0.37	0.39	0.35	0.40	0.33	0.43	0.31	0.684	0.012	
14:0	3.91	3.59	3.90	3.91	4.21	4.14	4.17	3.96	4.22	3.70	4.50	3.40	0.503	0.114	
<i>cis</i> -9 14:1	0.31	0.28	0.30	0.21	0.36	0.18*	0.37	0.16**	0.34	0.28	0.39	0.29	0.002	0.023	
15:0	0.11	0.11	0.10	0.10	0.11	0.10	0.10	0.10	0.10	0.11	0.12	0.10	0.823	0.004	
16:0	33.00	31.25	30.26	32.51	31.91	34.56	31.41	33.49	30.86	32.26	34.90	32.00	0.176	1.083	
<i>cis</i> -9 16:1	12.06	11.13	10.69	8.17*	11.74	6.94**	11.80	6.28***	10.57	10.06	12.20	11.02	0.011	0.695	
17:0	0.18	0.21	0.17	0.20	0.17	0.21	0.15	0.24	0.16	0.18	0.17	0.19	0.286	0.015	
18:0	3.46	3.84	3.31	4.65**	3.32	5.54***	3.08	5.48***	3.23	3.81	3.11	3.56	<0.001	0.168	
<i>cis</i> -9 18:1	27.83	29.23	30.17	29.83	29.87	29.01	29.65	30.21	31.61	30.21	27.44	30.42	0.845	1.358	
<i>cis</i> -11 18:1	1.77	1.99	1.80	1.88	1.82	1.78	1.81	1.90	1.90	1.85	1.68	1.91	0.449	0.079	
18:2	9.39	9.66	9.86	9.93	9.76	10.56	10.17	10.57	10.95	11.34	10.02	11.38	0.766	0.347	
18:3	0.79	0.79	0.84	0.81	0.83	0.85	0.88	0.82	1.05	1.01	0.93	1.00	0.774	0.040	
20:0	0.08	0.09	0.07	0.10***	0.07	0.12***	0.07	0.12***	0.07	0.09	0.08	0.09	<0.001	0.003	
<i>cis</i> -9, <i>trans</i> -11 18:2	0.08	0.09	0.10	0.09	0.09	0.08	0.10	0.09	0.10	0.09	0.07	0.09	0.798	0.012	
<i>cis</i> -5,8,11,14,17 20:4	0.34	0.34	0.32	0.31	0.34	0.35	0.32	0.33	0.32	0.35	0.29	0.34	0.697	0.009	
<i>cis</i> -5,8,11,14,17 20:5	0.13	0.12	0.12	0.11	0.14	0.13	0.13	0.14	0.15	0.14	0.12	0.12	0.371	0.008	
<i>cis</i> -(4,7,10,13,16,19) 22:6	0.22	0.21	0.19	0.21	0.23	0.25	0.21	0.24	0.21	0.23	0.20	0.22	0.450	0.011	

*p < 0.05, **p < 0.01, ***p < 0.001 in comparison with day 1 within treatment.

†Mean of samples from 4 and 1 day before treatment period started and immediately before treatments starts at day 1 in the treatment period.

‡p-values reported are for testing the interactions between treatments and time in the treatment period.

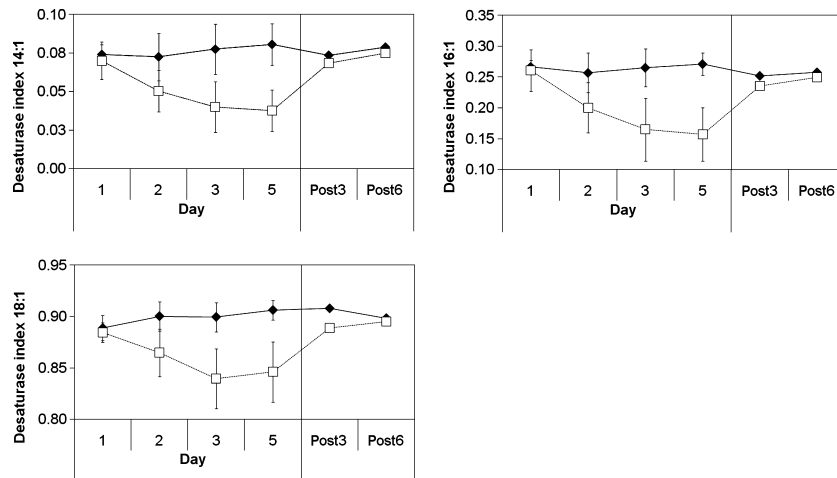


Fig. 1 Calculated desaturase indices (\pm SD) from sows' milk before (day 1), during (days 2–5) and after (post3 and post6) intramuscular injections of cobalt (—□—) or saline solution (—◆—). Values represent means from five sows. Desaturase indices from days 2, 3 and 5 were significant different ($p < 0.005$) from day 1 for sows having intravenous injections of cobalt.

period and for the sows in the control group during the treatment period. By contrast, the average content of Co in milk over the experimental period from sows treated with Co was $0.35 \pm 0.14 \mu\text{g/g}$. Cobalt-treated and control groups did not differ in milk concentrations of Fe, the values being 0.88 ± 0.34 vs. $0.74 \pm 0.074 \mu\text{g/g}$ respectively.

Feed intake and animal performance

The Co-treatment had a numerically but not statistically significant decreasing effect on *ad libitum* feed intake (7.8 kg/day vs. 9.0 kg/day , $p = 0.439$) over the 5-day treatment period. The Co-group had a numerically higher weight loss during the 8 days from day 1 of the treatment period (6.2 kg vs. 4.7 kg , $p = 0.608$). There was no effect of the Co-treatment on litter weight gain (data not shown).

Discussion

The effect of intramuscular injection of Co on Δ^9 -desaturase indices and milk fatty acid composition was highly significant (Fig. 1 and Table 3), but less evident than earlier obtained in bovine milk (Taugbøl et al., 2008, 2010). Thus, these results indicate that the role of Δ^9 -desaturase in udder fat metabolism is less in sows in relation to dairy cows. Polyunsaturated fatty acids have been shown to be the main dietary component regulating Δ^9 -desaturase (Ntambi, 1992, 1999; Ntambi et al., 1996; Sessler et al., 1996), suggesting that the disparity may be related to the difference in the supply of fatty acids

to the udder of sows, in relation to dairy cows. In dairy cows, as much as 70 to more than 90% of the unsaturated 18-carbon fatty acids are completely hydrogenated to 18:0 in the rumen (Doreau and Ferlay, 1994; Loor et al., 2004; Jenkins and Bridges, 2007). Accordingly, the supply to the udder of unsaturated fatty acids is much higher in sows in relation to dairy cows.

The reduction in Δ^9 -desaturase indices obtained by Co-treatment could be caused either by negatively affecting the synthesis of the enzyme, the activity of the enzyme, or both. The Δ^9 -desaturase enzyme system contains a di-iron centre in cytochrome b_5 , and it is known that Co interacts with Fe (Thomson et al., 1971; Flanagan et al., 1980). The Co-treatment reduced desaturase indices already 1 day after injection (Fig. 1); it therefore hypothesised that loosely bound Co directly interferes with the insertion of Fe into the di-iron centre, thereby reducing the enzyme activity in the mammary cells, as discussed by Taugbøl et al. (2010). However, regulation of mRNA expression can also occur rapidly. Jones et al. (1998) reported a considerable up regulation of Δ^9 -desaturase mRNA expression within 6 h of glucose treatment in 3T3-L1 adipocytes, a cell line that differentiates into an adipocyte-like phenotype under appropriate conditions. Hence, more investigation is needed to clarify the mechanisms behind these changes in milk fatty acid composition.

By contrast to the effect on milk, the Co-treatment had no effect on Δ^9 -desaturase indices of plasma lipids, with exception of *cis*-11 18:1 in plasma PL (Table 2). Lipid metabolism in sows is probably similar

to that in human, where adipose tissue lipid metabolism is unaffected by the lactation stage, provided there is a sufficient energy intake (Prentice and Prentice, 1988; Mcnamara, 1995). However, with a moderate restriction in feed intake, adipose tissue lipogenesis is reduced (Parmley et al., 1996). In the present experiment, the sows lost weight (in average 0.7 kg/day) during the experimental period and were obviously in a negative energy balance. Therefore, according to Parmley et al. (1996), the rate of lipogenesis and Δ^9 -desaturase mRNA can be expected to be considerably down-regulated in adipose tissue, explaining the lack of effect of the Co-treatment on the Δ^9 -desaturase indices of plasma lipids.

Gene expression of Δ^9 -desaturase is high in the lactating mammary gland and in subcutaneous adipose tissue in the goat and ovine (Ward et al., 1998; Barber et al., 2000; Bernard et al., 2005). Moreover, the effect on regulation of Δ^9 -desaturase mRNA or activity has been reported to be the same in mammary-, hepatic- and other tissues in the mouse (Ntambi, 1999; Lin et al., 2004). Provided there are similar mechanisms amongst species, this implies that the effect of Co-treatment on Δ^9 -desaturase in subcutaneous adipose- and hepatic tissue would be similar to that of the mammary gland. Another aspect of Co is its toxicity in sows. The EU legislation allows 2 mg Co/kg feed, but considers to reduce it further to 1 mg Co/kg feed (EFSA, 2009). This level is much lower than the upper level of 200 mg/kg feed before toxicological signs appear (Huck and Clawson, 1976). To our knowledge, there are no published results on the toxicological level of intramuscular injected Co in sows. Assumed that 5% of inorganic Co is absorbed, as found in human (Leggett, 2008), the upper level of 200 mg/kg feed as reported by Huck and Clawson (1976) correspond to 10 mg Co absorbed per kg feed. In present experiment, approximately 15 mg Co per kg feed was given as intramuscular injections. However, there were no obvious signs of toxic effects, but it has to be kept in mind that this was a short time study with the treatment period lasting for only 5 days.

Cobalt is a component of Co-ethylenediaminetetraacetic acid (Co-EDTA) which is commonly used as a liquid marker in digestibility experiments with pigs. Daily amounts in the range of 140–700 mg Co expressed per kg feed are reported from experiments with piglets and growing pigs (Turlington et al., 1989; Li et al., 1990; Jongbloed et al., 1992; Mroz et al., 1994; Corl et al., 2008). The extent of absorption of Co from Co-EDTA is not known in pigs, and it is therefore not possible to compare the amounts of Co

from Co-EDTA commonly used with the amounts administered intravenously in the present experiment. However, in dairy cows, orally administration of 3.5 g/day of Co (between 150 and 200 mg/kg feed) given as Co-EDTA affected milk desaturase indices (Taugbøl et al., 2008). Co-EDTA is therefore unsuitable as a liquid phase marker in experiments with dairy cows, at least when lipid metabolism is involved.

Different aspects of Co in pig nutrition need further investigation. Important questions which need to be explored are the extent of absorption of inorganic Co, whether Co-EDTA is suitable as a liquid phase marker in pigs where fat metabolism is a part of the study and at what levels Co influence fat metabolism in lactating and non-lactating animals.

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Paper II

The effect of excess cobalt on milk fatty acid profiles and transcriptional regulation of SCD, FASN, DGAT1 and DGAT2 in the mammary gland of lactating dairy cows

ORIGINAL ARTICLE

The effect of excess cobalt on milk fatty acid profiles and transcriptional regulation of SCD, FASN, DGAT1 and DGAT2 in the mammary gland of lactating dairy cows

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Keywords

cobalt, Δ^9 -desaturase, stearoyl-CoA desaturase, SCD, FASN, DGAT1, DGAT2, dairy cow

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Summary

The main objective of this study was to investigate the effect of excess cobalt (Co) on gene expression of stearoyl-CoA desaturase (SCD), fatty acid synthase (FASN), diacylglycerol acyltransferase 1 (DGAT1) and diacylglycerol acyltransferase 2 (DGAT2) of lactating dairy cows in relation to milk fatty acid profile. Seven multiparous cows of the Norwegian Red cattle breed (NRF) had their basal diet supplemented with 1.4 g Co as a 24 g/l solution of Co-acetate *per os* twice daily for 7 days followed by a 9-day depuration period. Udder biopsies were performed prior to the treatment period, after 1 week of treatment and immediately after the depuration period. Excess Co reduced the proportion of all *cis*-9 monounsaturated fatty acids and increased the proportion of 18:0 in milk. However, gene expression levels of SCD, DGAT1, DGAT2 and FASN were not significantly altered. Our results indicate that the effect of Co on milk fatty acid profile is mediated at the post-transcriptional level by reduced activity of SCD in the mammary gland. Potential mechanisms explaining how Co might reduce stearoyl-CoA desaturation are discussed.

Introduction

Stearoyl-CoA desaturase (SCD), also referred to as Δ^9 -desaturase, is the rate-limiting enzyme catalysing the synthesis of monounsaturated fatty acids from saturated fatty acids (Nakamura and Nara, 2004; Paton and Ntambi, 2009). Preferred substrates are 16:0 and 18:0, which are converted into *cis*-9 16:1 and *cis*-9 18:1 respectively (Ntambi, 1995). These fatty acids are major substrates for the synthesis of various lipids including phospholipids, triacylglycerols, cholesterol esters, wax esters and alkyldiacylglycerols. Thus, SCD is a central enzyme in lipid metabolism which is hypothesized to affect a variety of key physiological functions (Paton and Ntambi, 2009).

Dietary factors including glucose, fructose, vitamin A, cholesterol, vitamin D, polyunsaturated fatty acids, alcohol and conjugated linoleic acid are known to regulate SCD gene expression [reviewed by Ntambi and Miyazaki (2004)]. Excess iron (Fe) increases the SCD gene expression in the liver of mouse (Pigeon et al., 2001), while zinc (Zn) decreases the gene expression in the liver of rats (Sun et al., 2007). Recently, studies have demonstrated that Cobalt (Co) reduces the proportion of stearoyl-CoA desaturated fatty acids in milk when supplied in large amounts in both dairy cows (Shingfield et al., 2008; Taugbøl et al., 2008, 2010) and lactating sows (Karlengen et al., In press). However, the regulation of SCD is complex; no conclusions can be drawn from these past experiments to determine

whether Co depresses SCD at a transcriptional or post-transcriptional level or both.

Taugbøl *et al.* (2010) observed changes in Δ^6 -saturated fatty acids in plasma as well as in short-chain saturated fatty acids in the milk of dairy cows after administration of high Co doses. Therefore, Co may also affect fatty acid desaturase 2 (FADS2), also known as Δ^6 -desaturase, and fatty acid synthase (FASN) which is a multi-enzyme that plays a key role in *de novo* fatty acid synthesis (Wakil, 1989). In addition, diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT2) are enzymes catalysing the formation of triacylglycerols from diacylglycerols and acyl-CoA (Cases *et al.*, 1998). In this reaction, *cis*-9 18:1 (oleic acid) is the preferred substrate (Ntambi and Miyazaki, 2004). Thus, it can be speculated that DGAT1 and DGAT2 are affected by Co treatment because of a reduction in *cis*-9 18:1 as a substrate for these enzymes.

The onset of lactation results in a >40-fold up-regulation of mRNA for SCD and several other genes associated with lipid synthesis in mammary tissue of cows (Bionaz and Loor, 2008). Hence, SCD gene expression in lactating mammary tissue is normally high. The main objective of this experiment was to investigate the effect of excess Co on gene expression of SCD, as well as FADS2, FASN, DGAT1 and DGAT2 in relation to changes in milk fatty acid profiles of lactating dairy cows, by using real competitive PCR (rcPCR).

Material and methods

Animals, experimental design and treatment

The care and handling of the cows conformed to the laws and regulations controlling experiments with animals in Norway (The Animal Protection Act of 20, December 1974, and the Animal Protection Ordinance Concerning Experiments in Animals of 15, January 1996). Experimental animals were seven multiparous cows of the Norwegian Red breed (NRF) (654 ± 43 kg), averaging 286 ± 62 days in milk and 22 ± 3 kg milk/day at the start of the experiment. They were housed in a tie-stall barn and had free access to water. The experiment lasted 16 days; the first 7 days (day 1–7) were the treatment period while the last 9 days (day 8–16) were the depuration period. During the treatment period, the animals basal diet was supplemented *per os* 0.25 l of a 24 g/l solution of Co-acetate (Honeywell Speciality Chemicals, Seelze, Germany) at 07:30 and 15:00 hours, corresponding to 2.8 g Co/day. The basal diet consisted of pre-wilted grass silage, con-

taining 511, 44 and 151 g/kg dry matter (DM) of neutral detergent fibre (NDF), fat and protein, respectively, offered *ad libitum* starting 14 days before the treatment period. A commercial concentrate mixture (Table 1) was supplemented according to the cows calculated energy requirement and silage intake at the onset of the experiment. Daily amount of the concentrate mixture was divided into four equal portions and offered by an automatic feeder at 04:00, 06:00, 12:00 and 15:00 hours, which is the standard routine in the barn. Accidentally, the concentrate reserved for the experiment was given only 1 day before the experimental period and not from 14 days before as planned. However, this change in the concentrate given had no effect on milk content of the products and substrates for SCD in a parallel study with three dairy cows, without Co administration, practising the same feeding regime as in this experiment (results not shown).

Biopsy procedure

Biopsies were taken before the treatment period started (day 0), after 1 week of treatment (day 7)

Table 1 Ingredients composition and calculated chemical composition of the concentrate mixture (g/kg feed)

Ingredients	
Barley	540
Peas	120
Soybean meal, extracted	90
Sugar cane molasses	80
Rapeseeds	50
Oats	50
Corn gluten	30
Urea	9.0
Limestone powder	8.4
Salt	7.3
Monocalcium phosphate	5.2
Vegetable fat	5.0
Magnesium oxide	3.7
Mineral premix*	1.5
Sodium sulphate	1.5
Vitamin/taste premix†	0.6
Calculated contents	
Crude protein	186
Crude fat	38
NDF	139
Starch	364
Ash	59

*Containing per kg feed: copper [copper(II) sulphate], 15 mg; selenium (sodium selenite), 0.40 mg; zinc (zinc sulphate), 65 mg; iodine (calcium iodate), 2 mg; cobalt [cobalt(II) sulphate], 0.25 mg.

†Containing per kg feed: vitamin A, 5000 IU; vitamin D3, 2000 IU; vitamin E (alpha-tocopherol acetate), 50 mg.

and 9 days after the treatment was ended (day 16). All biopsies were taken after the morning milking. The cows were restrained in a head catch, moderately sedated with an intravenous injection of 0.8 ml of xylazine (Rompun 20 mg/ml; Bayer AG, Leverkusen, Germany), and their rear glands were shaved. A 15 × 20 cm area of the skin on the right or left rear gland was washed with water and sterilized with a chlorhexidine surgical scrub. An incision of the skin and gland capsule 1–2 cm in length was cut using a scalpel. Care was taken not to perforate blood vessels visible on the gland. The biopsy needle (Quick-Core® Biopsi Needle, 14 gage and 6 cm long; William Cook Europe, Bjaeverskov, Denmark) was locked into position and inserted through the skin incision with the specimen notch (20 mm) completely covered by the cutting canula. At the correct needle position, the stylet was advanced to expose the specimen notch, and the plunger of the cutting canula was depressed to remove a specimen, all according to the manufactures instructions (William Cook Europe). If the first sample did not contain an adequate amount of tissue, two biopsies were collected. After sampling, the incision was closed with one stitch and covered with Wound Plast Spray (Jørgen Kruuse AS, Langeskov, Denmark). Biopsies were collected in RNAlater™ (QIAGEN, Hilden, Germany) and stored at –80 °C until analysis.

Milk recording, sampling and analysis

Milk yields were recorded daily at 06:30 and 16:30 hours by the use of Tru-Test Milk Meter (Tru-Test Distributors, Auckland, New Zealand). In cases where the milk was contaminated with blood after the udder biopsies, cows were milked with a bucket milker and the milk was manually weighed. Aliquot milk samples were taken from the morning and evening milk at day-1 (before the treatment started) as well as at day 2, 3, 6, 8, 9, 10, 13 and 15. Samples were pooled to have one representative sample per cow per day. From each of these samples, an amount of 70 ml was preserved with 2-bromo-2-nitropropane-1,3-diol and stored at 4 °C until fat content analysis using an infrared milk analyzer (MilkoScan 6000; Foss Electric, Hillerød, Denmark). In addition, 20 ml of the samples withdrawn day 1, 6 and 15 were stored at –20 °C until fatty acid composition analysis. Milk lipids were extracted with ethanol, diethyl ether and petroleum ether (International IDF standard nr 1D: Milk Determination of Fat Content, Reference Method). The extracted lipids were methylated according to Kramer et al. (1997)

with NaOCH₃ and HCl/methanol. Fatty acid methyl esters were separated and quantified as described by Taugbøl et al. (2008).

Calculation of SCD indices

The SCD indices were calculated as (product of SCD)/(product of SCD + substrate of SCD) (Kelsey et al., 2003). The SCD indices were calculated for three pairs of fatty acids: *cis*-9 14:1–14:0, *cis*-9 16:1–16:0 and *cis*-9 18:1–18:0.

Expression analysis

Total RNA was purified and treated with DNase using the RNeasy Lipid Tissue Mini Kit (QIAGEN). First-strand cDNA synthesis was conducted using SuperScript™-II Rnase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT primer T270. From each tissue, 0.5 µg of total RNA was used as template.

An assay was designed for concurrent transcript profiling of *SCD*, *FADS2*, *FASN*, *DGAT1* and *DGAT2* by rcPCR as described by the manufacturer (SEQUENOM, San Diego, CA, USA). Competitors at known concentrations and cDNA were coamplified with primers SCD-F, SCD-R, FADS2-F, FADS2-R, FASN-F, FASN-R, DGAT1-F, DGAT1-R, DGAT2-F and DGAT2-R in one reaction, followed by an extension reaction using primers SCD_E, FADS2_E, FASN_E, DGAT1_E and DGAT2_E (Table 2). The experiment was run with two parallel PCR for each tissue. Estimated expression levels were imported from the software MASSARRAY QGE 3.4 (SEQUENOM).

Table 2 List of oligonucleotides used in the gene expression analysis

Primer ID	5'-sequence-3'
DGAT1-F	ACGTTGGATGAGAACAGCTTGAGGAAGAGG
FASN-F	ACGTTGGATGAAGAAGGAAGCGTCAAACCG
FADS2-F	ACGTTGGATGGCATTGAGTACCAGGAGAAG
SCD-F	ACGTTGGATGTTTATCCGACCTAAGAGCCG
DGAT2-F	ACGTTGGATGAAGCAGCTCCAAGTCATCTC
DGAT1-R	ACGTTGGATGCCTTCTCCTCGAGTCTATC
FASN-R	ACGTTGGATGATGATGACAGCGGTGGAAG
FADS2-R	ACGTTGGATGACAGCTGCCAGACTTCCTC
SCD-R	ACGTTGGATGGAAGCACAACAACAGGACAC
DGAT2-R	ACGTTGGATGTGTACATGAGGATGACGCTG
DGAT1-E	CTATCACTCCAGTGGGC
FASN-E	GGTGAAGGCCGACTAT
FADS2-E	CTTCTCAGGGACCCGATG
SCD-E	CACCAGTTTGTAGTACCTCCTC
DGAT2-E	GACGCTGCAGGCCACTCCCAGCACG

Statistical analysis

MATLAB version 7.9, R2009b (Mathworks, Natick, MA, USA) was used for the statistical analysis of the gene expression data. For each gene, the log fold change (equivalent to log ratio) between day 0 and 7 (FC21), 7 and 16 (FC32) and 0 and 16 (FC31) were calculated as:

$$FC_{ijk} = \log(E_{ij}) - \log(E_{ik})$$

where E_{ij} and E_{ik} is the estimated cDNA concentration for time point j and k for animal i respectively. Ordinary t -tests were used to test if the log fold changes were different from zero for each gene separately.

All other parameters were analysed by the use of analysis of variance using the mixed procedure of SAS software version 9.1 (SAS Institute, Cary, NC, USA). The measurements were repeated several times for each animal and were found to be correlated. Consequently, this correlation was taken into account in the statistical model by including animal in the model as a random effect. A covariance structure of the repeated measurements was chosen by comparing potential structures using Akaike's and Schwarz's Bayesian information criterion (Wolfinger, 1996). First-order autoregressive structure proved useful for all data. Analysis of variance for repeated measurements was performed according to the following model: $Y_{ijk} = \mu + A_i + d_j + \varepsilon_{ijk}$ where μ is the intercept, A_i the fixed effect of time, d_j the random effect of cow and ε_{ijk} represents the experimental error. Differences between means for the different sampling points were estimated by the least squares means.

Results

There were no consistent changes in milk fat yield during the experiment (Fig. 1). However, milk fat yield decreased slightly, but not significantly ($p > 0.05$), the first day after the udder biopsies. The effect of excess Co on fatty acid composition and desaturase indices in milk is presented (Table 3). Excess Co reduced all *cis*-9 monounsaturated fatty acids and increased 18:0 in milk, resulting in decreased desaturase indices ($p < 0.001$) from 0.11 to 0.05 for *cis*-9 14:1, from 0.04 to 0.02 for *cis*-9 16:1 and from 0.69 to 0.51 for *cis*-9 18:1. Moreover, milk fat proportion of *cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3 significantly increased ($p < 0.05$), whereas *cis*-6, 9, 12 18:3, *cis*-9, *trans*-11

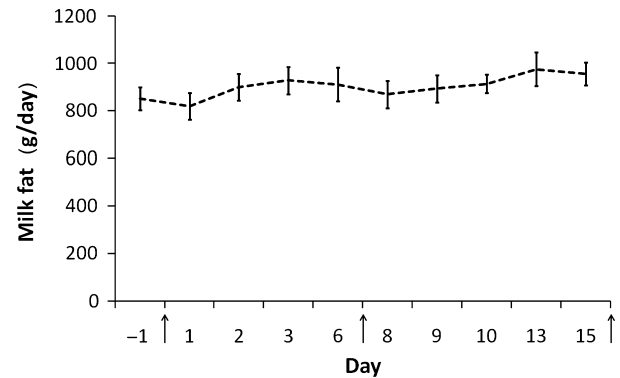


Fig. 1 Milk fat production (\pm SE) at start of the experiment (day-1), during the treatment period (day 1–7) and during the post-treatment period (day 8–15). Arrows at the x-axis indicates the time for udder biopsies.

18:2, *cis*-5, 8, 11, 14 20:4 and *cis*-5, 8, 11, 14, 17 20:5 significantly decreased ($p < 0.05$). It is also noteworthy that the proportion of 4:0 increased from 4.16 to 4.83 g/100 g FA ($p < 0.001$), whereas the proportion of 8:0 and 12:0 decreased from 1.46 to 1.29 ($p = 0.008$) and 3.71 to 3.06 ($p = 0.041$) g/100 g FA respectively. Milk fatty acid composition was not completely recovered 8 days after the treatment was ended (day 15). As a consequence of this, desaturase indices of 14:1, 16:1 and 18:1 were significantly lower at day 15 compared to day-1 ($p < 0.001$), but significantly higher than day 6 ($p = 0.008, 0.003$ and < 0.001 for desaturase indices of 14:1, 16:1 and 18:1 respectively).

The gene FADS2 had low, unreliable expression levels and was therefore excluded from the results. Two animals had missing observations for gene expression on day 0 because of inadequate amounts of tissue from the udder biopsies. The SCD gene expression was not significantly affected by excess Co (Fig. 2a). However, the variation was substantially higher at day 0 and day 16 compared to day 7. This was because of deviating low values for SCD gene expression for one animal on day 0 and another animal on day 16. The same animals had corresponding low values for gene expression of FASN which was not statistically affected ($p > 0.05$) by excess Co (Fig. 2d). The expression of DGAT1 and DGAT2 was not affected by excess Co (Fig. 2b,c). However, DGAT2 tended ($p = 0.07$) to be differentially expressed between day 7 and day 16 (Fig. 2c). Also, DGAT2 had large variations in gene expression between animals, especially at day 0 and 7.

Discussion

The ratio of saturated to monounsaturated fatty acids affects phospholipid composition, and alteration in

Table 3 Proportions of milk fatty acids and desaturase indices (DI) before (–1), after 6 days of Co treatment and 8 days after the Co treatment ended (day 15). Values represent means from seven cows*

Sampling day	–1	6	15	SEM	p-value†
Fatty acids, g/100 g fatty acids					
4:0	4.16 ^c	4.83 ^a	4.42 ^b	0.084	<0.001
6:0	2.52	2.50	2.57	0.068	0.679
8:0	1.46 ^a	1.29 ^b	1.45 ^a	0.073	0.008
10:0	3.25	2.84	3.22	0.239	0.069
<i>Cis</i> -9 10:1	0.38 ^a	0.14 ^c	0.30 ^b	0.020	<0.001
12:0	3.71 ^a	3.06 ^b	3.61 ^a	0.275	0.041
14:0	12.55	12.78	12.79	0.359	0.616
<i>Cis</i> -9 14:1	1.55 ^a	0.61 ^c	0.94 ^b	0.100	<0.001
15:0	1.12	1.07	1.07	0.032	0.074
16:0	30.88	32.21	32.02	0.750	0.317
<i>Cis</i> -9 16:1	1.43 ^a	0.61 ^c	0.94 ^b	0.078	<0.001
17:0	0.54 ^b	0.60 ^a	0.53 ^b	0.014	<0.001
<i>Cis</i> -9 17:1	0.18 ^a	0.11 ^c	0.13 ^b	0.007	<0.001
18:0	7.75 ^c	12.78 ^a	9.73 ^b	0.337	<0.001
<i>Trans</i> -9 18:1	0.19 ^a	0.17 ^b	0.16 ^b	0.008	0.006
<i>Trans</i> -10 18:1	0.27 ^a	0.26 ^a	0.22 ^b	0.014	0.015
<i>Trans</i> -11 18:1	1.34	1.77	1.56	0.106	0.417
<i>Cis</i> -9 18:1	17.10 ^a	13.13 ^c	15.14 ^b	0.541	<0.001
<i>Cis</i> -11 18:1	0.51 ^b	0.61 ^a	0.49 ^b	0.015	<0.001
<i>Cis</i> -9, 12 18:2	1.03 ^b	1.09 ^a	1.11 ^a	0.054	0.025
<i>Cis</i> -6, 9, 12 18:3	0.05 ^a	0.03 ^b	0.04 ^{ab}	0.003	0.017
<i>Cis</i> -9, 12, 15 18:3	0.70 ^c	0.89 ^b	0.95 ^a	0.032	<0.001
20:0	0.14 ^b	0.18 ^a	0.18 ^a	0.007	0.002
<i>Cis</i> -9, <i>trans</i> -11 18:2	0.75 ^a	0.43 ^c	0.61 ^b	0.028	<0.001
<i>Cis</i> -5, 8, 11, 14 20:4	0.06 ^a	0.05 ^b	0.05 ^b	0.003	0.006
<i>Cis</i> -8, 11, 14, 17 20:4	0.07 ^b	0.07 ^b	0.08 ^a	0.003	0.023
<i>Cis</i> -5, 8, 11, 14, 17 20:5	0.07 ^a	0.05 ^b	0.07 ^a	0.004	<0.001
<i>Cis</i> -4,7,10,13,16,19 22:6	0.008	0.008	0.008	0.001	0.627
DI 14:1	0.11 ^a	0.05 ^c	0.07 ^b	0.007	<0.001
DI 16:1	0.04 ^a	0.02 ^c	0.03 ^b	0.002	<0.001
DI 18:1	0.69 ^a	0.51 ^c	0.61 ^b	0.009	<0.001

*Different letters within row indicate significant differences ($p < 0.05$).

†p-values reported are for testing the effect of time.

this ratio has been implicated in overall energy metabolism and a variety of disease states (Ntambi and Miyazaki, 2004). Accordingly, the finding that Co alters bovine milk fatty acid composition is of great interest. The amount of Co administered in the present study (150 mg/kg diet DM) is higher than the maximum tolerable level, set to 25 mg Co/kg DM (NRC, 2005). This threshold level is mainly based on experiments with calves (Ely et al., 1948; Keener et al., 1949), and it appears that young calves are more sensitive to Co compared to older calves (Keener et al., 1949). It is also suggested that Co absorption decline with age in other species (Naylor and Harrison, 1995). Controlled studies to assess Co tolerance in older cattle have, as far as we know, not been reported. However, adult male sheep tolerated as much as 180 mg Co/kg DM in an experiment lasting for 109 days without any symptoms of toxicity (Corrier et al., 1986). Comparable

studies with Co (Shingfield et al., 2006, 2008; Taugbøl et al., 2008, 2010), or digestibility experiments with comparable amounts of Co administered as cobalt-ethylene-diaminetetraacetic acid (Co-EDTA) (Volden, 1999; Shingfield et al., 2003; Stevnebo, 2009), have not indicated any signs of toxicity. This indicates that the effects in the present study are of general physiological relevance rather than specific dysfunctions caused by Co overload.

The gene expression array used in this study did not include any housekeeping genes. As the measurements were repeated at the same individuals, the biological variation between individuals is to a certain extent accounted for. In addition, all genes were run in a single multiplex, and possible experimental variation between samples should be similarly reflected in all analysed genes. The individual variations at the different time points do not indicate a such systematic effect.

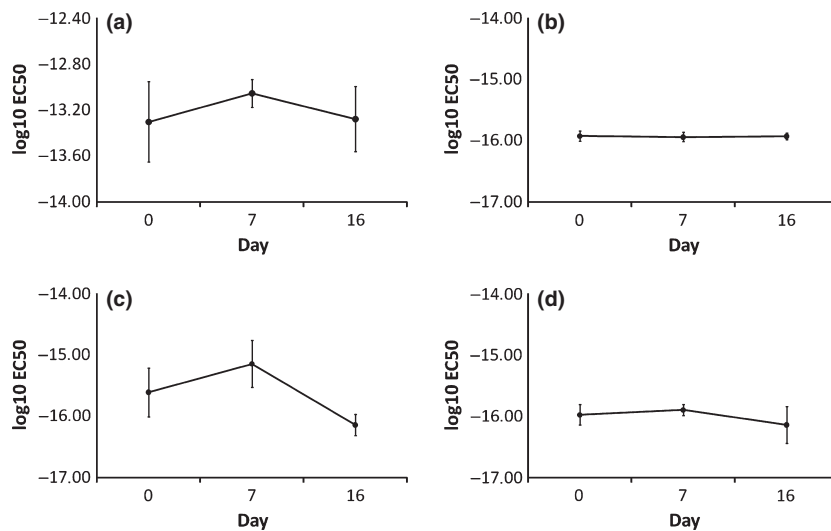


Fig. 2 Gene expression (\pm SE) of SCD (a), DGAT1 (b), DGAT2 (c) and FASN (d) before Co treatment (day 0), after 7 days of Co treatment and 9 days after Co treatment ended (day 16).

In this experiment, administration of 2.8 g Co/cow/day had similar effects on milk fatty acid composition and the resultant desaturase indices as administration of 3.5 g in the experiments of Taugbøl *et al.* (2008, 2010). Furthermore, excess Co had no significant effect on the gene expression level of SCD. To our knowledge, these are the first results demonstrating the effect of Co on gene expression of SCD in the mammary gland. Minerals can alter gene expression in hepatic tissue; excess Fe increases the gene expression of SCD in mice (Pigeon *et al.*, 2001) while Zn excess decreases SCD gene expression in rat (Sun *et al.*, 2007). In addition to gene expression levels, minerals have been shown to affect SCD enzyme activity. For instance, the SCD enzyme activity increases with Fe overload (Pigeon *et al.*, 2001) and decreases with cadmium (Cd) overload (Kudo *et al.*, 1991). On the other hand, the effect of copper (Cu) is unclear as it is reported to both increase (Elliot and Bowland, 1968; Ho and Elliot, 1973) and decrease (Sreekrishna and Joshi, 1980) SCD activity. Deficiencies of minerals can also alter SCD activity. An increase in SCD activity is observed in situations with Zn deficiency (Clejan *et al.*, 1981; Cunnane and Wahle, 1981), while deficiency of Fe (Rao *et al.*, 1980, 1983) and Cu (Wahle and Davies, 1974, 1975) decrease SCD activity. Moreover, interactions between Cd and Zn (Kudo *et al.*, 1991), and possibly between Cu and Zn (Cunnane, 1982), could also affect SCD activity. Therefore, the regulation of SCD by minerals is complex.

In the present study, there were some indications of reduced expression or activity of some other desaturases. Milk fat proportion of *cis*-6, 9, 12 18:3

depends on Δ^6 -desaturase activity, whereas the milk fat proportion of *cis*-5, 8, 11, 14 20:4 and *cis*-5, 8, 11, 14, 17 20:5 depends on both Δ^5 - and Δ^6 -desaturase activity. Because the proportion of these particular fatty acids decreased and their corresponding substrates (*cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3) increased, we hypothesize that Co reduced Δ^6 -desaturase activity and possibly also Δ^5 -desaturase activity in the bovine mammary gland. These results are similar to those of Taugbøl *et al.* (2010), who found indications of reduced Δ^6 -desaturation in dairy cows during administration of high Co doses, based on the composition of both plasma fatty acids and milk fatty acids. NADH-cytochrome b5 reductase and cytochrome b5 are both part of the SCD enzyme system in conjunction with the terminal desaturase (Ntambi, 1995). Like SCD, Δ^6 -desaturase is dependent on cytochrome b5 as an electron donor in animals (Tocher *et al.*, 1998). Cytochrome b5 may therefore represent a site of inhibition for both Δ^6 -desaturase and SCD.

Co-ions are able to induce *de novo* synthesis of haem oxygenase, the rate-limiting enzyme of haem degradation, in liver, kidney and other tissues (Maines and Kappas, 1974, 1975, 1976a,b). As cytochrome b5 is a haemoprotein, we can speculate that Co induces the synthesis of haem oxygenase which results in a decrease in cytochrome b5 and a subsequent reduction in desaturase activity. The transfer of electrons from cytochrome b5 to SCD is central in the oxidation–reduction reactions required for the conversion of acyl-CoA substrates to monounsaturated fatty acids (Dailey and Strittmatter, 1980; Mitchell and Martin, 1995). However, functional

studies are required to understand the molecular interactions mediated by Co on the fatty acid desaturation process.

The tendency of a higher gene expression of DGAT2 at day 7 compared to day 16 indicates that Co might have a positive effect on expression of this gene. DGAT2 is located in close proximity to SCD in the endoplasmic reticulum (ER) (Man et al., 2006). In general, the fatty acids 16:0 and 18:0 from the diet or *de novo* synthesis are desaturated to 16:1 and 18:1 by SCD and then channelled to DGAT2 for the final step in triacylglycerol synthesis in the ER (Man et al., 2006). Thus, we can speculate that DGAT2 is regulated as a consequence of the reduction of easily available substrate caused by the reduced amount of products from SCD (16:1 and 18:1). However, because of the lack of significance differences in DGAT2 expression levels between time points of this experiment, more results are needed to confirm the effect of Co on this gene. Despite the increased proportions of 4:0 and reduced proportions of 8:0 and 12:0 during Co excess, no changes in gene expression of FASN were found. Accordingly, the mechanisms explaining the effects on these fatty acids are not known. The rationality of these effects is also unknown, but one hypothesis is that this is a mechanism to compensate for the higher amount of saturated fatty acids to keep membranes fluid as the melting point of 4:0 is low (-7.9°C).

The present results and findings of others (Shingfield et al., 2008; Taugbøl et al., 2008, 2010) show that Co has some interesting effects on fat metabolism in the mammary gland of dairy cow. Our study indicates that this effect of Co is mediated at a post-transcriptional level by reduced activity of SCD in the mammary gland. For further studies, it would be interesting to measure the amount of cytochrome b5 and the level of bilirubin, to determine whether haem oxygenase is induced. In addition, the activity of enzymes responsible for fatty acid synthesis and fatty acid desaturation should be examined, especially SCD, FASN and FADS2. It would also be of great interest to examine the effect of Co excess on fatty acid desaturation in other lipogenic tissues such as hepatic and adipose tissue.

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Paper III

**Effect of different levels of supplied cobalt on the fatty acid
composition og bovine milk**

Effect of different levels of supplied cobalt on the fatty acid composition of bovine milk

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Key words: Δ^9 -desaturase, milk fat, cobalt, dairy cow

Abstract

In previous studies, administration of high amounts of cobalt (Co) decreased the proportion of MUFAs in bovine milk. The present study was conducted to examine the amount of Co needed to obtain this effect. High yielding dairy cows ($n = 4$), equipped with ruminal cannulas, were used in a 4 x 4 Latin square design study. The basal diet consisted of concentrate mixture (9 kg/d) without added Co, and grass silage (*ad libitum*). Four levels of Co were administered as Co-acetate dissolved in distilled water; No Co (T1), 4.0 mg Co/d (T2), 360 mg Co/d (T3), and 5,100 mg Co/d (T4). During treatment periods, the solutions were continuously infused into the rumen. Milk yield and milk concentration of fat, fatty acids (FAs), protein, lactose, Co, zinc, iron, and copper were determined. Blood plasma was analyzed with respect to FAs, Co, zinc, iron, and copper. Feed intake and total tract digestibility of feed components were also determined. With some few minor exceptions, only the highest level of Co (T4) affected FA composition. In general, the effects of Co on FA composition in blood were insignificant compared to the effects on milk. In milk fat, the concentration of oleic acid was reduced as much as 38% on T4 compared with T1 (12.9 vs. 20.7 g/100 g FA, respectively). The highest Co level also depressed feed intake and milk yield. It is concluded that normal variations in Co intake may have no significant effect on the level of MUFAs in milk fat.

Introduction

The bovine milk fat fraction is generally characterized by a low concentration of unsaturated fatty acids (FAs)⁽¹⁾. Oleic acid (*cis*-9 18:1) is the unsaturated FA with the highest concentration, accounting for 20-30% (w/w) of the total FAs⁽²⁾. The concentration of *cis*-9 FAs in bovine milk is determined by their supply to the udder and by the extent of desaturation of their corresponding saturated FAs caused by the action of Δ 9-desaturase (stearoyl-CoA desaturase) in the mammary gland⁽³⁾. In previous studies, large amounts of cobalt (Co) produced a reduction in the concentration of MUFAs in bovine milk^(4,5,6,7,8). Large amounts of Co might also affect the proportions of some PUFAs in milk fat⁽⁴⁾. In those experiments, the supplied Co was present in different physico-chemical forms (Co-EDTA or Co-acetate) and different methods of administration were used (*per os*, intraruminal, or intravenous). However, in all those studies, the concentration of Co given was as high as 50 to 150 mg/kg diet dry matter (DM). That level of Co is very high compared to the dietary requirement for Co (0.11 mg Co/kg DM) set for dairy cows⁽⁹⁾, and even compared to the maximum tolerance level of 25 mg Co/kg feed DM set for cattle (NRC, 2005).

From a human health perspective, a lowered concentration of unsaturated FAs in milk and milk products is undesirable ⁽¹⁰⁾. Thus, the main objectives of the present experiment were to determine if the commonly used level of Co in the diets of dairy cows reduces milk concentration of MUFAs, and secondly, to determine how much Co is needed to produce this effect.

Materials and methods

Animals, experimental design, feeds, feeding, treatments, and feed sampling

Three cows of the Norwegian red cattle breed in their second and one in its fourth lactation, weighing 649 ± 39 kg, averaging 89 ± 15 d in milk, (DIM), and yielding 34.5 ± 2.5 kg/d of milk at the start of the experiment were used for this study. All cows were equipped with ruminal cannulas (Bar Diamond Inc., Parma, ID, USA; 100 mm i.d.). They were housed in individual tie stalls and had free access to water. The care and handling of cows conformed to the laws and regulations controlling experiments with live animals in Norway (The animal protection act of December 20, 1974 and the animal protection ordinance concerning experiments in animals of January 15, 1996.)

The experiment was designed as a 4 x 4 Latin square with 4 treatments, 4 lactating cows, and 4 periods. Each period lasted for 18 d, and was divided into a treatment period of 11 d (day 1-11) and a post-treatment period of 7 d (days 12-18). The basal diet consisted of a concentrate mixture without extra Co (Table 1) plus grass silage, mainly prewilted timothy and meadow fescue ensiled with a formic acid based additive. All cows were fed the same amount of concentrate (9 kg/d), close to their individual requirement at the start of the study, and amounts were held constant throughout the study. The concentrate was offered in 3 equal meals at 06.00, 14.00, and 22.00 h. The grass silage was given *ad libitum*. Representative samples of the feeds were taken daily and composed to one representative sample of concentrate and one representative sample of silage per period. Feed refusals were removed and recorded daily before the morning feeding. The treatments included 4 levels of cobalt (Co): basal diet plus distilled water (no added Co) (Treatment 1, T1), T1 plus 4.0 mg Co per day (T2), T1 plus 360 mg Co per day (T3), and T1 plus 5,100 mg Co per day (T4). The concentrations of Co in the concentrate and silage were 0.3 and 0.2 mg/kg DM, respectively (Table 1), resulting in a Co concentration of ~0.24 mg/kg DM in the total diet with T1. In T2, the amount of Co given was comparable to (slightly above) the level normally supplied when using a commercial concentrate mixture in Norway. The Co levels in T3 and T4 were ~100 and 1,000-fold

this level, respectively. T4 was ~20% above the level reported to affect desaturase indices in earlier experiments ^(7,8).

Cobalt was given as Co-acetate ($C_4H_6CoO_4 \cdot 4H_2O$) from Honeywell Speciality Chemicals (Seelze, Germany) dissolved in distilled water. The solutions were continuously infused through plastic pipes into the rumen during the entire treatment period, using a peristaltic pump at a rate of ~ 2.3 litres per day. Representative samples of the different Co solutions were taken each period before the treatment started.

Feces and urine collection, and sampling

Faeces and urine from cows on T2 and T4 were collected separately for 72 h, starting at 08.00 h on day 8. Faeces were collected in steel containers located under the back of the cows, whereas urine was collected through tubes that were attached to the cows and led into plastic containers located next to the containers for feces. Both urine and feces were collected and weighted 3 times per 24 h, at 14.00, 22.00, and 08.00 h. To prevent ammonia from evaporating, 500 mL of sulphuric acid (1 M H_2SO_4) was added to the urine cans before starting and after each emptying. The collected feces and urine were stored in plastic buckets at 4°C. After 24 h of collection, urine and faeces were carefully blended before representative samples for analysis were taken out.

Blood sampling

Blood samples were taken from cows on T2 and T4 on days 11 and 18. The samples for FA analysis (5 mL heparin-containing tubes) were withdrawn from the milk vein at 08.00, 10.00, and 12.00 h. The samples for analysis of Co, Fe, Cu, and Zn (10 mL heparin-containing tubes) were taken from the jugular vein at 10.00 h. Blood was centrifuged at $500 \times g$ for 20 minutes and plasma was stored at -20°C until analysis.

Milk recording and sampling

The cows were milked and yields recorded by use of a Tru-Test Milk Meter (Tru-Test Distributors Ltd., Auckland, New Zealand), twice daily (at 06.30 and 16.30 h), throughout the entire study. Milk samples were collected on days 1, 2, 3, 5, 8, 11, 12, 13, 15, and 18, and a 70 mL aliquot from each

day was preserved with 2-bromo-2-nitropropane-1,3-diol and stored at 4°C until analysis of fat, protein, lactose, and urea. Non-preserved milk samples (20 mL aliquot) from each day were used for analysis of FA composition, and samples (10 mL aliquot) collected on days 11 and 18 were used for analysis of concentrations of Co, Fe, Cu, and Zn. These samples were kept frozen (-20°C) until analysis.

Chemical analysis

Feeds were analyzed for DM and ash according to Malkomesius and Nehring⁽¹¹⁾, and neutral detergent fiber (NDF) was analyzed according to Mertens et al.⁽¹²⁾. Crude protein (CP = Kjeldahl-N · 6.25) was analyzed according to the AOAC method 985.13⁽¹³⁾ with the following modifications: Samples were digested with 15 mL H₂SO₄, 3.5 g K₂SO₄, and 0.4 g CuSO₄, boiled for 45 minutes at 420°C and 25 mL of distilled water was added after cooling. Analysis of starch was performed according to McCleary et al.⁽¹⁴⁾, and crude fat (CF) was analyzed after hydrolysis with petroleum ether on an Accelerated Solvent Extractor (ASE200) from Dionex (Sunnyvale, CA, USA).

Samples of feed for analyses of Co, Fe, Zn, and Cu were pretreated according to the following procedure: Dried feed (1 g) was accurately weighed into a MLS-Milestone Ultra Clave III Teflon digestion vessel (Mikrowellen Labor Systeme GmbH, Leutkirch, Germany), and 5 mL of concentrated Ultrapure nitric acid and 5 mL MQ water (18Ω) were added to the vessel. A known concentration of a Yttrium standard solution was added as a yield monitor. The system was closed, loaded with nitrogen to 140 bars, and the mixture was heated to 250° C for 30 min. The samples were then transferred to beakers and diluted with MQ water (18Ω) to a volume of 50 mL. Then, the concentrations of Fe, Co, Zn, and Cu were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin-Elmer Optima 5300 DV Inductively Coupled Plasma Optical Emission Spectrometer (Shelton, CT, USA), equipped with a standard torch and a Perkin Elmer AS 93⁺ Autosampler. The Optima 5300 DV is a simultaneous ICP-OES instrument with an echelle polychromator and a segmented array charge-coupled detector. The determination of Fe was based on the 238.204 nm line, Co on the 228.616 nm line, Zn on the 206 nm line, and Cu on the 327.393 line. The limits of detection were defined as 10* SD of 10 blank samples, and were 0.02, 0.06, 0.07, and 0.05 µg/g for Co, Zn, Fe, and Cu, respectively. The concentrations of Co in the Co-solutions were determined by ICP-OES as described for feed, but without any pre-treatment. Faeces were analyzed for DM, ash, NDF, CP, starch, and CF as described for feed.

Analysis of blood plasma FAs was performed as described by Taugbøl et al. ⁽⁷⁾. For determination of Fe, Co, Zn, and Cu, 3-5 mL of plasma was accurately weighed into a MLS-Milestone Ultra Clave III Teflon digestion vessel (Mikrowellen Labor Systeme GmbH, Leutkirch, Germany), and samples were then pre-treated and analyzed for Fe, Co, Zn, and Cu using ICP-OES as described for feed.

Analyses of fat, protein, lactose, and urea concentration of milk were performed using an infrared milk analyzer (MilkoScan 6000; Foss Electric, Hillerød, Denmark). Milk lipids were extracted with ethanol, diethyl ether, and petrol ether (International IDF standard nr 1D: Milk Determination of Fat Content, Reference Method). The extracted lipids were methylated according to Kramer et al. ⁽¹⁵⁾ with NaOCH₃ and HCl/methanol. Fatty acid methyl esters were separated and quantified according to Taugbøl et al. ⁽⁷⁾. For determination of Fe, Co, Zn, and Cu, 5 mL of milk was accurately weighed into a Teflon digestion vessel (MLS-Milestone Ultra Clave III (Mikrowellen Labor Systeme GmbH, Leutkirch, Germany), and samples were then pre-treated and analyzed for Fe, Co, Zn, and Cu as described for feed.

Calculations

Feed intake was calculated as the difference between the feed given and the refusals collected before morning feeding the following day for 3 consecutive days (days 9-11) in each period. Milk yield was calculated as the mean of the yield measured for 3 consecutive days (days 9-11). Production of fat, protein, and lactose was calculated from the average milk yield on days 9-11, multiplied by the analyzed concentration of fat, protein, and lactose, on day 11. The amount of Co administered per day was calculated as the product of the average amount of solution infused between days 6-9 and the analyzed concentration of Co in the solutions. Total tract digestion of feed components was calculated as the difference between their average intake for days 6-9 and their average fecal excretion on days 8-10. The $\Delta 9$ -desaturase indices were calculated as: [product of $\Delta 9$ -desaturase] / [product of $\Delta 9$ -desaturase + substrate of $\Delta 9$ -desaturase]. The $\Delta 9$ -desaturase indices were calculated for 3 pairs of FAs: *cis*-9 14:1-14:0, *cis*-9 16:1-16:0, and *cis*-9 18:1-18:0. The desaturase indexes for $\Delta 6$ -desaturase and $\Delta 5$ -desaturase in plasma were calculated as [*cis*-6,9,12 18:3] / [*cis*-6,9,12 18:3 + *cis*-9,12 18:2], and [*cis*-5,8,11,14 20:4] / [*cis*-5,8,11,14 20:4 + *cis*-8,11,14 20:3], respectively.

Statistical analysis

The data were analyzed as a Latin square using analysis of variance with the mixed procedure of Statistical Analysis Systems statistical software package version 9.1 (SAS Institute, Cary, NC, USA). The following model was used to analyze the data:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + \varepsilon_{ijk}$$

where μ is the general mean, α_i is the random effect of a cow, β_j is the effect of treatment, δ_k is the effect of period, and ε_{ijk} represents the experimental error. For parameters with repeated measurements within a cow and period (feed intake and milk yield), cow within period were treated as repeated, and a first-order autoregressive structure AR(1) was used as the covariance structure. The level for statistical significance was defined as $P=0.05$, and $0.1 > P > 0.05$ was considered to indicate a tendency of effect. All data are presented as means with their standard errors unless otherwise stated.

Results

Fatty acid composition and desaturase indices in blood plasma and milk

Proportions of plasma FAs and calculated desaturase indices are presented in Table 2. In general, increasing Co supplementation from T2 (4.0 mg/d) to T4 (5,100 mg/d) had relatively small effects on plasma FA composition. Of the saturated FAs, 17:0 was significantly reduced, whereas the others were unaffected. Oleic acid (*cis*-9 18:1) tended to be depressed (5.49 vs. 6.18 g/100 g FAs; $P=0.053$), whereas the proportion of *cis*-11 18:1 tended to increase (0.26 vs. 0.23 g/100 g FAs; $P=0.091$). However, the sum of monounsaturated FAs (MUFAs) decreased significantly. The effect of Co level on the proportion of the polyunsaturated FAs (PUFAs) was not consistent. The plasma concentration of *cis*-6, 9, 12 18:3 tended to be reduced (0.52 vs. 0.97 g/100 g FAs; $P=0.068$) when cows received T4, resulting in a tendency for a lower $\Delta 6$ -desaturase index (0.01 vs. 0.03; $P=0.060$). Also, the plasma concentration of *cis*-8, 11, 14 20:3 (1.01 vs. 1.54 g/100 g FAs; $P=0.011$) decreased. In contrast, *cis*-5, 8, 11, 14 20:4 (1.09 vs. 1.04 g/100 g FAs; $P=0.024$) increased slightly, resulting in a tendency for an increased $\Delta 5$ -desaturase index (0.53 vs. 0.40; $P=0.057$).

As expected, the FA composition of plasma on T4 had, with only a few exceptions, reached the T2 level the last day of the post-treatment period (day 18) (data not shown). However, on day 18, *cis*-5, 8, 11, 14, 17 20:5 was significantly ($P=0.042$) lower on T4 compared to T2, and some other FAs

also tended to be affected. In contrast to day 11, neither the concentration of MUFAs nor the resultant desaturase indices were significantly different between T4 and T2 at day 18.

In contrast to plasma, milk FA composition was highly affected by treatment (Table 3). With a few exceptions, only the highest level of Co (T4) significantly differed from the other treatments. Concentrations of all *cis*-9 MUFAs (product FAs of Δ 9-desaturase) in milk were significantly decreased with T4 compared to the other treatments ($P \leq 0.001$), resulting in significantly lower desaturase indices for 14:1 ($P < 0.001$), 16:1 ($P = 0.002$), and 18:1 ($P < 0.001$). The effects on their corresponding saturated FAs were not consistent, but the concentration of 18:0 was 24.91 g/100 g FAs on T4 compared to 15.35 g/100 g FAs on T2; representing an increase of > 60% (w/w) ($P < 0.001$). Interestingly, and in contrast to *cis*-9 MUFA, the concentrations of the essential FAs *cis*-9, 12 18:2 and *cis*-9, 12, 15 18:3 (substrate FAs for Δ 6-desaturase) increased ($P < 0.05$). However, the concentrations of *cis*-9, *trans*-11 18:2 (CLA) (product of Δ 9-desaturase) and *cis*-5, 8, 11, 14, 17 20:5 (EPA) (product of Δ 6-desaturase and Δ 5-desaturase) were lowered ($P < 0.001$). The concentration of 17:0 in milk increased when cows received T4. In addition, cows on T4 had a lower concentration of FAs with chain lengths < 16 carbon atoms ($P < 0.001$), indicating reduced de novo FA synthesis. With the exception of 4:0 which tended to increase ($P = 0.051$), all FAs with chain lengths up to 14 C-atoms decreased ($P \leq 0.001$) on T4.

With a few exceptions, T2 and T3 had insignificant effects on milk FA composition. However, the concentration of *cis*-9 14:1 was significantly ($P < 0.05$) lower on T3 (0.62 g/100 g FAs) than on T1 (0.77 g/100 g FAs), and the desaturase index of 14:1 was significantly ($P < 0.05$) lower on T1 (0.06) than on T2 (0.05) and T3 (0.05). Moreover, the concentration of 17:0 was significantly ($P < 0.05$) higher on T2 (0.43 g/100 g FAs) and T3 (0.42 g/100 g FAs) compared to T1 (0.40 g/100 g FAs).

The FA composition in milk was not completely recovered the last day of the post-treatment period (day 18) with T4 (data not shown). Accordingly, most FAs still showed significant ($P < 0.05$) differences between T4 and the other treatments, resulting in significantly ($P < 0.05$) different desaturase indices (Figure 1). However, the effects were less evident, and there were no differences between T1, T2, and T3. In contrast to day 11, the FAs 16:0 ($P = 0.018$) and *cis*-5, 8, 11, 14 20:4 ($P = 0.017$) were significantly different between T4 and the other treatments (data not shown).

Co, Fe, Cu, and Zn in blood plasma and milk

Blood plasma and milk concentrations of Co, Zn, Fe, and Cu are shown in Figures 2 and 3, respectively. There were interesting differences between T4 and T2 for some of these parameters. Compared with T2, cows on T4 produced milk with a higher concentration of Fe (0.19 vs. 0.13 mg/kg; $P=0.014$), but lower concentration of Zn (3.84 vs. 4.68 mg/kg; $P=0.002$). Parallel effects were found in blood plasma, but were not statistically significant ($P>0.1$). The amounts of Zn and Fe excreted in milk were not different ($p>0.05$), but the excretion of Zn tended to be reduced with T4 (98.22 vs. 140.41 mg/d; $P=0.076$). The concentrations of Co, Fe, Zn, and Cu in plasma and milk were not significantly different ($P>0.05$) between T4 and T2 at day 18. However, the concentration of Co in plasma and milk, and its excretion in milk (data not shown) at day 18 still tended to be higher with T4 than with T2. Moreover, at day 18, the concentration of Fe in plasma and Cu in milk tended to be higher with T4 than T2.

Feed intake, diet digestibility, and milk production

The highest level of Co (T4) significantly depressed silage intake and total DM intake compared with T1, T2, and T3 ($P=0.001$ and $P=0.002$, respectively) (Table 4). However, Co level had no significant effects on the digestibility of the main diet nutrients (Table 5). Due to the negative effect of T4 on grass silage intake, the amount of protein digested per day was significantly lower on T4 compared to T2 (2,468 vs. 2,737 g/d; $P=0.008$), and there was a similar tendency for the amount of digested fat (1,224 vs. 1,334 g/d; $P=0.071$).

In line with the negative effect on feed intake, milk yield was depressed on T4 compared to the other treatments ($P=0.001$) (Table 4). However, this negative effect of T4 on milk yield was temporary; occurring from day 6 to day 15 (results not shown). In contrast to the milk yield, milk concentrations of fat, protein, and lactose were not affected by the treatment. However, as a consequence of the reduced milk yield, production of fat, protein, and lactose were significantly reduced ($P=0.044$, $P=0.006$, and $P=0.040$, respectively) when cows received T4 (Table 4). However, production of fat was not significantly different ($P>0.05$) between T2 and T4.

Discussion

The main objective of the present study was to determine how much Co is required in the diet for dairy cows to change their milk FA composition. Earlier studies⁽⁶⁾, showed that the ratio between

products and substrates for $\Delta 9$ -desaturase stabilized after 6 d of Co-infusion; to be certain of this effect, we therefore planned a treatment period of 11 d. As shown in Figure 1, the response to Co levelled off before the treatment ended for desaturase indexes of *cis*-9 14:1, and *cis*-9 16:1; but for *cis*-9 18:1, no conclusion could be drawn. When the present experiment was planned, no published results on the time required for recovery of milk FA composition after Co administration were available. The depuration period of 7 d was obviously too short, because desaturase indices did not level off at the end of the depuration period (Figure 1). A parallel effect on Co concentration in blood plasma and milk support those results (Figures 2 and 3). In a study published later by Shingfield et al. ⁽⁵⁾, it was shown that the ratio between products and substrates for $\Delta 9$ -desaturase in bovine milk did not level off 5 d after terminating the Co-infusion. Data from 2 cows changing diets from T4 to T2 (in the present study) showed that desaturase indices seemed to level off at ~11th day on T2, corresponding to 18 d after Co infusion was ended on T4 (data not shown). Therefore, as the experiment was not designed to adjust for eventual carry-over effects, it is not clear whether the minor effects of T2 vs. T1 on FA composition are effects of the treatment or carry-over effects from T4. It does appear that T3 had a slight reducing effect on desaturase indices, at least for *cis*-9 14:1 (Table 3, Figure 1). However, overall results clearly show that large amounts of Co are required to obtain significant effects on FA composition in bovine milk. With exception of the desaturase index for *cis*-9 14:1 and the concentration of *cis*-9 14:1 and 17:0 in milk, supplementation with as much as 360 mg/d Co (T3) did not affect desaturase indices or individual FA proportions. However, addition of 5,100 mg/d Co (T4) significantly affected milk FA composition and resulted in considerable decreases in $\Delta 9$ -desaturase indices in milk, which is in line with other studies ^(4,5,6,7,8). Besides affecting desaturase indices for even numbered FAs, Co infused into the rumen also increased the concentration of 17:0 in milk. However, this is probably not a consistent effect, because Shingfield et al. ⁽⁵⁾ found no effect of infusing Co into the rumen on the concentration of 17:0 in bovine milk. In other studies ^(4,5,6,7,8), the effects of Co on desaturase indices were comparable with the results obtained in the present study, even when using a lower level of Co than T4, but a considerably higher level of Co than for T3. Based on the present results and the literature ^(4,5,6,7,8), the required level of Co for obtaining significant effects on milk FA composition is probably in the range of 20 – 50 mg/kg feed DM.

NRC ⁽¹⁶⁾ set the maximum tolerable level of Co at 25 mg/kg diet DM, which is probably in the lower range of levels required for affecting the FA composition. In our study, the highest level of Co (270 mg Co /kg DM) depressed intake of silage and lowered milk yield (Table 4). Accordingly, as discussed by Karlengen et al. ⁽⁴⁾, the level of 270 mg Co/kg DM may have some adverse effects. .

However, in the study conducted by Shingfield et al. ⁽⁵⁾ as well as Karlengen et al. ⁽⁴⁾, using administration of 51 and 150 mg Co/kg DM, respectively, the treatment had no effect on feed intake or milk yield, even though the level of Co supplied was considerably higher than what is considered as the maximum tolerable level (NRC, 2005).

The Co requirement for ruminants is set at 0.11 mg/kg DM ⁽⁹⁾. With the concentrations of 0.2 mg/kg DM in the silage and 0.3 mg/kg DM in the concentrate used in the present study, there was surplus of Co even on the unsupplemented diet (T1). Norwegian commercial concentrate mixtures for dairy cows normally contain Co at levels comparable with T2. In addition, cows are often given Co-containing mineral supplements. Consequently, the intake of Co is, in most practical situations, considerably higher than the requirement of 0.11 mg/kg DM ⁽⁹⁾, but much lower than that required to significantly affect milk FA composition.

Our results showed only minor and, with a few exceptions, non-significant effects of Co on desaturase indices in plasma (Table 2). These results are in agreement with those of other investigators ^(7,8), and indicate that the mammary gland is the major site for $\Delta 9$ -desaturation during lactation in ruminants. A decrease in expression of $\Delta 9$ -desaturase in adipose tissue ⁽¹⁷⁾ considerably up-regulated the $\Delta 9$ -desaturase expression in the mammary gland ⁽¹⁸⁾, and an inactive $\Delta 9$ -desaturase in ruminant liver ⁽¹⁹⁾ supports this key role of the mammary gland for FA desaturation during lactation. Nevertheless, the plasma concentration of MUFA was slightly reduced with T4, indicating that Co, in principle, has effects in other tissues similar to those in mammary tissue.

Recent results ⁽⁴⁾ indicate that the effect of Co on bovine milk FA composition is caused by affecting $\Delta 9$ -desaturase activity in the udder rather than its production. However, the mechanisms behind the reductions in desaturation products when excess Co is given are not fully understood. Different minerals are reported to affect $\Delta 9$ -desaturase ^(20,21,22,23,24), and an indirect effect of Co on the $\Delta 9$ -desaturation cannot be excluded. Because excess Co tended to decrease plasma desaturase indices for $\Delta 6$ -desaturase and increase plasma desaturase indices for $\Delta 5$ -desaturase, a common mechanism for inhibition of $\Delta 9$ -desaturase and $\Delta 6$ -desaturase, but not $\Delta 5$ -desaturase, is suggested. Both $\Delta 6$ -desaturase and $\Delta 5$ -desaturase differ from $\Delta 9$ -desaturase by containing a fused cytochrome b5 domain at their N-terminus, which plays a role as an electron donor during desaturation ⁽²⁵⁾, and microsomal cytochrome b5 is essential for the activity of $\Delta 9$ -desaturase ^(26,27). However, microsomal cytochrome b5 also plays an important role in the process of $\Delta 6$ -desaturation ⁽²⁸⁾. The predicted amino acid sequence of $\Delta 5$ -desaturase contains all of the structural characteristics present in $\Delta 6$ -desaturase ⁽²⁹⁾, but a potential role of microsomal cytochrome b5 in the process of $\Delta 5$ -

desaturation has, to our knowledge, not been investigated. Cytochrome b5 is a hemoprotein ⁽³⁰⁾, and Karlengen et al. ⁽⁴⁾, suggests that an induction of heme oxygenase due to high amounts of Co may cause reduced $\Delta 9$ -desaturation. An induction of heme oxygenase can possibly affect the desaturation process by releasing heme-iron from cytochrome b5, and might therefore represent a site of inhibition for both $\Delta 9$ -desaturase and $\Delta 6$ -desaturase. The increased concentration of Fe in milk ($P < 0.05$) and the numerically higher concentration of Fe in plasma found when cows were on the highest amount of Co, might strengthen this theory. However, more investigation is needed to confirm this hypothesis.

In conclusion, adding 5,100 mg/d of Co significantly reduced the concentration of MUFAs, and increased the concentration of SFAs in milk fat, indicating reduced activity of $\Delta 9$ -desaturase. There were also indications of reduced $\Delta 6$ -desaturase activity. Adding such high amounts of Co to the diet reduced feed intake and milk yield. Based on the present results and the literature, the amount of Co needed to reduce the concentration of MUFAs in bovine milk fat is probably between 20 and 50 mg Co/kg diet DM, which is in the order of 100-200 times the normal intake of lactating dairy cows. Thus, in practice, the normal variation in Co intake may have no significant effect on milk FA composition.

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Table 1. Ingredient composition of the concentrate mixture and chemical composition of the concentrate mixture and grass silage.

	Concentrate mixture	Grass silage
<u>Ingredient composition (g/kg):</u>		
Oats	200	
Cane molasses	50	
Rapeseed meal	50	
Barley	423	
Sobean meal	50	
SoyPass®*	100	
Bran	50	
Soybean oil	20	
Fat†	30	
Limestone meal	5.5	
Mono-calcium phosphate	7.0	
Sodium chloride	5.0	
Magnesium oxide	4.0	
Mineralpremix‡	5.0	
Vitamins	0.5	
Dry matter (g/kg)	877	273
<u>Chemical composition (g/kg DM):</u>		
Crude protein	188	151
Starch	392	-
NDF	178	515
Fat	82	74
Ash	67	80
<u>Minerals (mg/kg DM):</u>		
Co	0.3	0.2
Zn	27	127
Fe	166	130
Cu	6.8	21.2

* Borregaard Ligno Tech, Sarpsborg, Norway.

† Aarhus Karlshamn Sweden AB, Sweden. Containing 920 g stearic acid per kg.

‡ Norsk Mineralnæring, Hensmoen, Hønefoss, Norway. Containing per kg feed: Copper (copper(II) sulphate); 15 mg, Selenium (sodium selenite); 0.25 mg, zinc (zinc sulfate); 65 mg, iodine (calcium iodate); 2 mg, manganese (manganese(II) sulfate); 30 mg.

Table 2. Effect of cobalt level supplied on proportions of plasma fatty acids and calculated desaturase indices at the last day of cobalt administration (day 11) (Mean values with their standard errors, n=4)

	T2	T4	SEM
<u>Fatty acid composition (g/100 g</u>			
<u>FA):</u>			
14:0	0.77	0.79	0.072
15:0	0.48	0.49	0.020
16:0	7.61	7.46	0.172
<i>cis</i> -9 16:1	0.79	0.87	0.036
17:0	0.51 ^a	0.45 ^b	0.029
18:0	12.01	11.87	0.342
<i>cis</i> -9 18:1	6.18 ^(a)	5.49 ^(b)	0.143
<i>cis</i> -11 18:1	0.23 ^(b)	0.26 ^(a)	0.008
<i>cis</i> -9, 12 18:2	35.09	36.82	0.715
<i>cis</i> -6, 9, 12 18:3	0.97 ^(a)	0.52 ^(b)	0.043
<i>cis</i> -9, 12, 15 18:3	7.32	8.34	0.284
22:0	0.26	0.26	0.019
<i>cis</i> -8, 11, 14 20:3	1.54 ^a	1.01 ^b	0.199
<i>cis</i> -5, 8, 11, 14 20:4	1.04 ^b	1.09 ^a	0.059
23:0	0.46	0.49	0.027
<i>cis</i> -8, 11, 14, 17 20:4	0.77	0.60	0.054
<i>cis</i> -5, 8, 11, 14, 17 20:5	0.99	0.97	0.073
24:0	0.50	0.51	0.030
<i>cis</i> -15 24:1	0.49	0.48	0.022
<i>cis</i> -7, 10, 13, 16, 19 22:5	0.64	0.67	0.011
<i>cis</i> -4, 7, 10, 13, 16, 19 22:6	3.24	3.33	0.047
SFA*	22.60	22.33	0.443
MUFA†	7.70 ^a	7.10 ^b	0.138
PUFA‡	51.60	53.34	0.819
<u>Desaturase indices (DI):</u>			
DI <i>cis</i> -9 16:1	0.09	0.10	0.003
DI <i>cis</i> -9 18:1	0.34	0.32	0.007
DI <i>cis</i> -6, 9, 12 18:3	0.03 ^(a)	0.01 ^(b)	0.001
DI <i>cis</i> -5, 8, 11, 14 20:4	0.40 ^(b)	0.53 ^(a)	0.025

T1, cows supplemented 4 mg Co per day; T4, cows supplemented 5,100 mg Co per day.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

^{(a),(b)} Mean values within a row with unlike superscript letters within brackets tended to be different ($0.1 > P > 0.05$)

* SFA = 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 22:0 + 23:0 + 24:0

† MUFA = *cis*-9 16:1 + *cis*-9 18:1 + *cis*-11 18:1 + *cis*-15 24:1

‡ PUFA = *cis*-9, 12 18:2 + *cis*-6, 9, 12 18:3 + *cis*-9, 12, 15 18:3 + *cis*-8, 11, 14 20:3 + *cis*-5, 8, 11, 14, 17 20:5 + *cis*-8, 11, 14, 17 20:4 + *cis*-5, 8, 11, 14, 17 20:5 + *cis*-7, 10, 13, 16, 19 22:5 + *cis*-4, 7, 10, 13, 16, 19 22:6

Table 3. Effect of cobalt level supplied on proportions of milk fatty acids and calculated desaturase indices at the last day of cobalt administration (day 11) (Mean values with their standard errors, $n=4$)

	T1	T2	T3	T4	SEM
<u>Fatty acid composition (g/100 g</u>					
<u>FA):</u>					
4:0	4.43 ^(b)	4.46 ^(b)	4.52 ^(b)	4.87 ^(a)	0.131
6:0	2.51 ^a	2.49 ^a	2.54 ^a	2.15 ^b	0.095
8:0	1.43 ^a	1.39 ^a	1.43 ^a	1.04 ^b	0.085
10:0	2.96 ^a	2.90 ^a	2.98 ^a	2.15 ^b	0.189
12:0	3.20 ^a	3.11 ^a	3.18 ^a	2.28 ^b	0.199
14:0	11.35 ^a	11.42 ^a	11.47 ^a	10.60 ^b	0.148
<i>cis</i> -9 14:1	0.77 ^a	0.64 ^{ab}	0.62 ^b	0.18 ^c	0.055
15:0	0.97	1.01	0.97	0.97	0.031
16:0	25.33	26.17	25.35	26.37	0.901
<i>cis</i> -9 16:1	0.77 ^a	0.70 ^a	0.69 ^a	0.35 ^b	0.050
17:0	0.40 ^c	0.43 ^b	0.42 ^{bc}	0.50 ^a	0.009
18:0	14.59 ^b	15.35 ^b	15.59 ^b	24.91 ^a	0.724
<i>trans</i> -9 18:1	0.19	0.18	0.18	0.18	0.004
<i>trans</i> -10 18:1	0.31	0.30	0.30	0.32	0.011
<i>trans</i> -11 18:1	1.30	1.38	1.32	1.41	0.066
<i>cis</i> -9 18:1	20.66 ^a	19.40 ^a	19.90 ^a	12.89 ^b	0.650
<i>cis</i> -11 18:1	0.27	0.26	0.26	0.31	0.017
<i>cis</i> -9, 12 18:2	1.29 ^b	1.32 ^b	1.27 ^b	1.53 ^a	0.066
<i>cis</i> -9, 12, 15 18:3	0.41 ^b	0.42 ^b	0.42 ^b	0.54 ^a	0.022
20:0	0.18 ^b	0.19 ^b	0.19 ^b	0.29 ^a	0.009
<i>cis</i> -9, <i>trans</i> -11 18:2	0.51 ^a	0.49 ^a	0.47 ^a	0.23 ^b	0.027
<i>cis</i> -5, 8, 11, 14 20:4	0.06	0.06	0.05	0.05	0.007
<i>cis</i> -5, 8, 11, 14, 17 20:5	0.05 ^a	0.04 ^a	0.04 ^a	0.03 ^b	0.003
<i>cis</i> -4, 7, 10, 13, 16, 19 22:6	0.007	0.007	0.006	0.007	0.0005
SFA*	67.22 ^b	68.74 ^b	68.45 ^b	75.84 ^a	0.755
MUFA†	22.47 ^a	21.00 ^a	21.47 ^a	13.72 ^b	0.734
PUFA‡	1.81 ^b	1.85 ^b	1.79 ^b	2.16 ^a	0.084
<u>Desaturase indices (DI):</u>					
DI 14:1	0.06 ^a	0.05 ^b	0.05 ^b	0.02 ^c	0.004
DI 16:1	0.03 ^a	0.03 ^a	0.03 ^a	0.01 ^b	0.002
DI 18:1	0.59 ^a	0.56 ^a	0.56 ^a	0.34 ^b	0.018

T1, cows supplemented 0 mg Co per day; T2, cows supplemented 4 mg Co per day; T3, cows supplemented 360 mg Co per day; T4, cows supplemented 5,100 mg Co per day.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0.05$)

* SFA = 4:0 + 6:0 + 8:0 + 10:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0

† MUFA = *cis*-9 14:1 + *cis*-9 16:1 + *cis*-9 18:1 + *cis*-11 18:1

‡ PUFA = *cis*-9, 12 18:2 + *cis*-9, 12, 15 18:3 + *cis*-5, 8, 11, 14 20:4 + *cis*-5, 8, 11, 14, 17 20:5 + *cis*-4, 7, 10, 13, 16, 19 22:6

Table 4. Effect of cobalt level supplied on feed intake, milk production, chemical composition, and production of fat, protein, and lactose (Mean values with their standard errors, $n=4$)

	T1	T2	T3	T4	SEM
<u>DM intake (kg/d):</u>					
Grass silage	13.3 ^a	13.2 ^a	12.9 ^a	11.2 ^b	0.68
Concentrate	7.9	7.8	7.9	7.8	0.07
Total	21.2 ^a	21.0 ^a	20.8 ^a	19.0 ^b	0.71
<u>Milk production:</u>					
<i>Yield (kg/d)</i>	31.6 ^a	30.9 ^a	32.5 ^a	26.6 ^b	1.40
<i>Composition(g/kg)</i>					
Fat	44.0	39.9	41.5	40.0	2.32
Protein	31.8	31.6	31.6	31.6	0.52
Lactose	46.2	46.8	46.4	47.0	0.76
<i>Production (g/d)</i>					
Fat	1373 ^a	1235 ^{ab}	1350 ^a	1050 ^b	77.8
Protein	999 ^a	973 ^a	1028 ^a	837 ^b	37.9
Lactose	1455 ^a	1443 ^a	1517 ^a	1252 ^b	68.8

T1, cows supplemented 0 mg Co per day; T2, cows supplemented 4 mg Co per day; T3, cows supplemented 360 mg Co per day; T4, cows supplemented 5,100 mg Co per day.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0.05$)

Table 5. Effect of supplied cobalt level on total tract digestion coefficients of feed ingredients (Mean values with their standard errors, $n=4$)

	T2	T4	SEM
Dry matter	0.761	0.749	0.008
Ash	0.683	0.650	0.015
NDF	0.743	0.715	0.007
Crude protein	0.761	0.742	0.011
Fat	0.803	0.806	0.010
Starch	0.994	0.995	0.002

T1, cows supplemented 4 mg Co per day; T4, cows supplemented 5,100 mg Co per day; NDF, neutral detergent fiber.

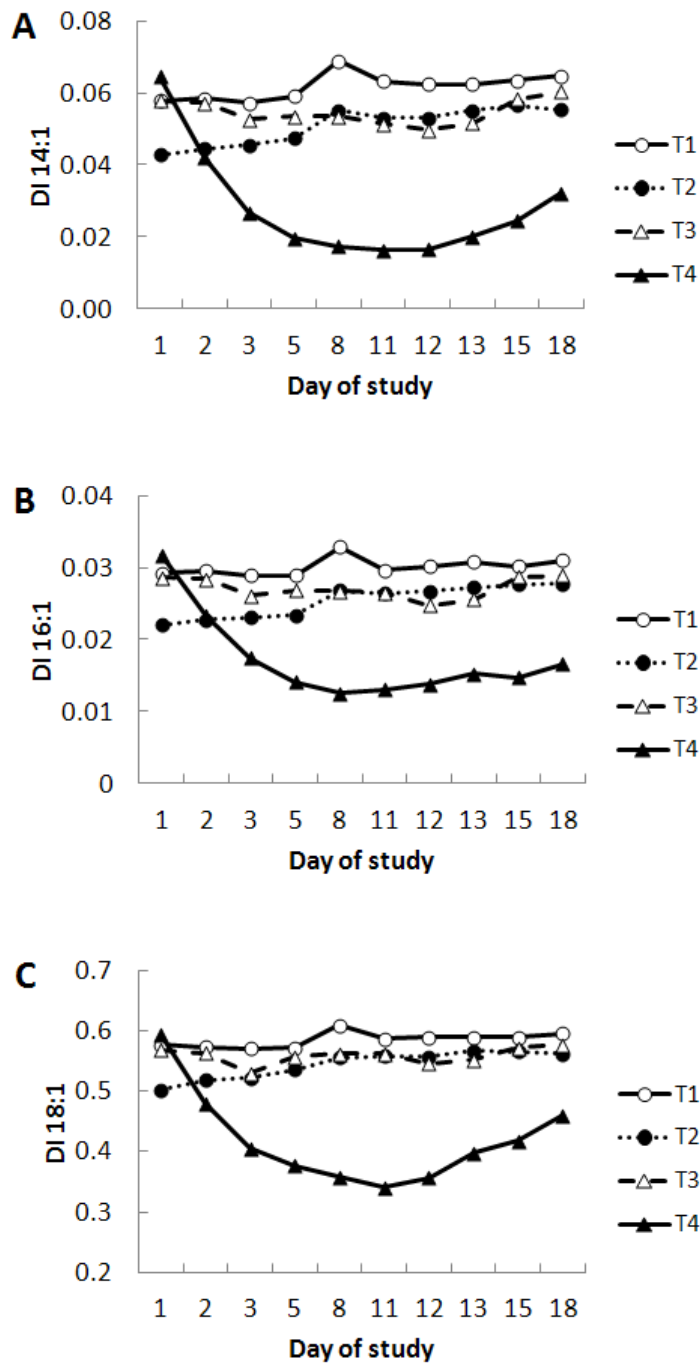


Figure 1. Temporal changes in milk desaturase indices (DI) for 14:1 (A), 16:1 (B), and 18:1 (C) in response to supplementation of different levels of Co during the treatment period (days 1 to 11) and the depuration period (days 12 to 18) ($n=4$). T1, cows supplemented 0 mg Co per day; T2, cows supplemented 4 mg Co per day; T3, cows supplemented 360 mg Co per day; T4, cows supplemented 5,100 mg Co per day.

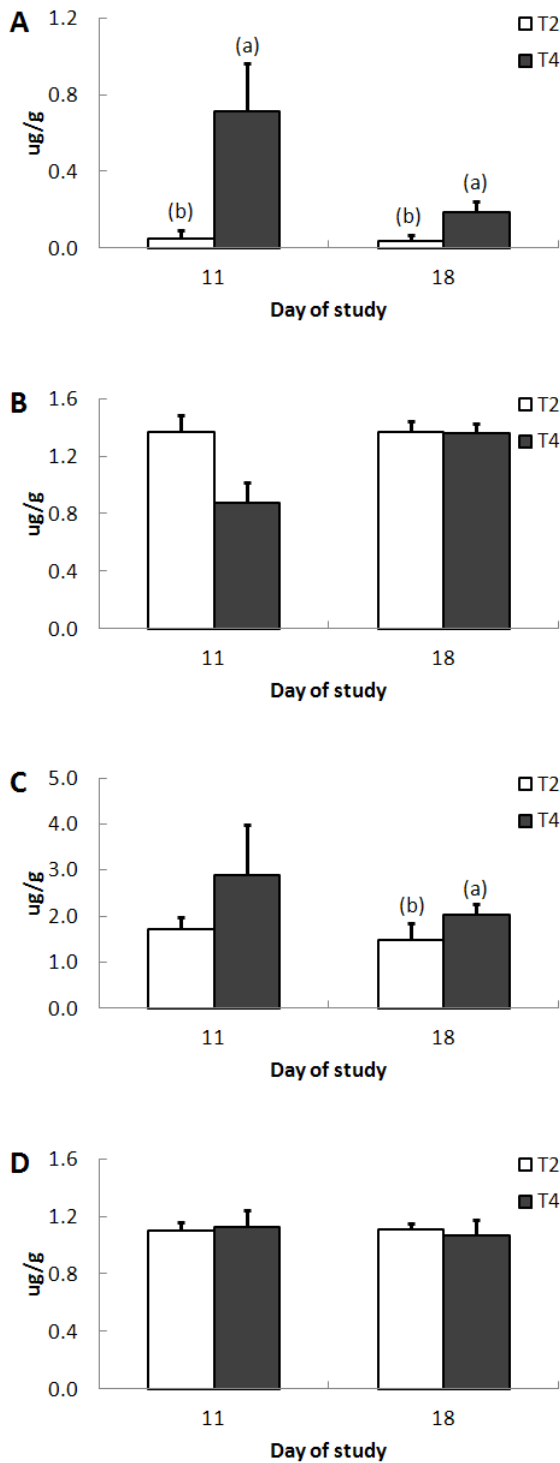


Figure 2. Concentration of cobalt (A), zinc (B), iron (C), and copper (D) in blood plasma the last day of the treatment period (day 11) and the last day of the depuration period (day 18) for cows supplemented with different levels of Co. Values are mean with their standard deviations represented by vertical bars ($n = 4$). ^{(a),(b)} Mean values with unlike superscript letters within brackets tended to be different ($0.1 > P > 0.05$). T2, cows supplemented 4 mg Co per day; T4, cows supplemented 5,100 mg Co per day.

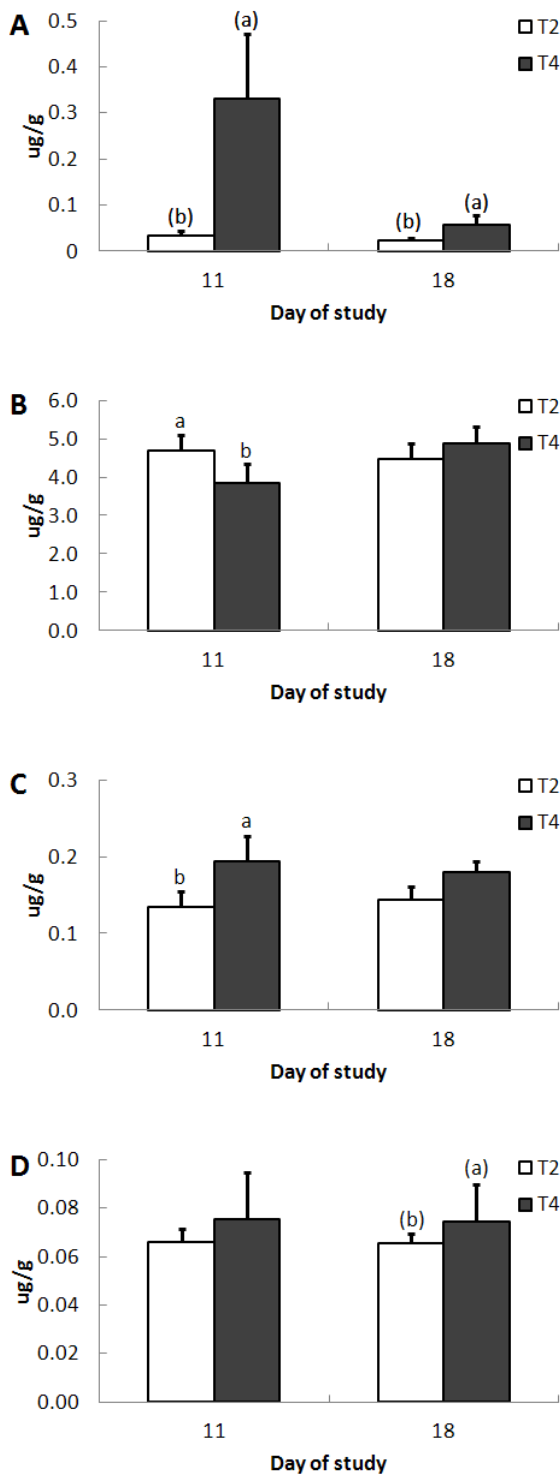


Figure 3. Concentration of cobalt (A), zinc (B), iron (C), and copper (D) in milk the last day of the treatment period (day 11) and the last day of the depuration period (day 18) for cows supplemented with different levels of Co. Values are mean with their standard deviations represented by vertical bars ($n = 4$). ^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$). ^{(a),(b)} Mean values with unlike superscript letters within brackets tended to be different ($0.1 > P > 0.05$). T2, cows supplemented 4 mg Co per day; T4, cows supplemented 5,100 mg Co per day.