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Selenite and selenate affected the fatty acid profile in *in vitro* incubated ovine ruminal fluid containing linoleic acid

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ABSTRACT

The influence of adding selenite (Se^{IV}) or selenate (Se^{VI}) to ovine ruminal fluid containing linoleic acid (LA) on the profile of fatty acids, especially conjugated linoleic acid (CLA) isomers and their metabolites was investigated. Dietary LA is incorporated by rumen bacteria, isomerized to other geometric and positional isomers, metabolized into CLA isomers, biohydrogenated to *trans*-vaccenic acid (TVA) and finally to C18:0. Considering the above, ovine ruminal fluid was incubated *in vitro* at 39°C under CO_2 either alone (the control ruminal fluid) or with a combination of LA (1.67 mg/ml), a low (0.167 μg/ml) or high (1.67 μg/ml) level of selenium as Se^{IV} or Se^{VI}. Tubes with examined ruminal fluid were removed after 0, 6, 12, 18, and 24 hrs of incubation and then submitted for determination of fatty acids (FA). FA, as methyl esters, were quantitated using capillary gas chromatography and flame-ionization detection. Both concentrations of Se^{IV} added to the ruminal fluid with LA usually decreased the concentrations of individual CLA isomers, especially *cis9trans11*CLA (*c9t11*CLA) and the sum of all CLA isomers in the ruminal fluid in comparison with the fluid containing only LA. Our studies documented that Se^{IV} reduced the capacity of bacterial isomerase, which turns the *cis9*-bond into a *trans10*-bond. The addition of Se^{IV} to the ruminal fluid with LA decreased the concentration of TVA compared with the fluid with only LA; a decrease in the loss of TVA was observed with increasing concentrations of Se^{IV}. The presence of Se^{IV} in the ovine fluid with LA stimulated the biohydrogenation of TVA to C18:0. The addition of LA to the incubated fluid, irrespectively of the presence of Se^{IV}, increased the concentration of C20:5n-3. Se^{VI} in the ruminal fluid with LA usually more efficiently increased the concentration of *c9t11*CLA, *t10c12*CLA, *c9c11*CLA and *t9t11*CLA, from 6 until 24 hrs of incubation compared with the fluid containing LA, regardless of the presence of Se^{IV}. The concentration of TVA in the fluid containing

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Se^{VI} and LA is higher than in the fluid with Se^{IV} and LA. Se^{VI} in the fluid increased the concentration of C18:0. As a consequence, Se^{VI} added to the fluid increased the yield of final biohydrogenation to C18:0 compared with the fluid with LA, irrespective of the presence of Se^{VI}. Further studies are required to clarify the effects of other Se-compounds and fatty acids on concentrations of fatty acids, especially CLA isomers and their precursors, in the ruminal fluid.

KEY WORDS: ruminal fluid, CLA isomers, fatty acid, linoleic acid, selenium, sheep

INTRODUCTION

Analysis of the selenium (Se) concentration of ruminal microbes from sheep fed natural diets revealed that ruminal microbial Se abundance was enriched relative to the concentration of Se in a diet. This concentration of Se in microbes was significantly higher than that of the diet, whether considered relative to diet dry matter (average of 46-fold), nitrogen (average of 11.3-fold), or sulphur abundance (average of 26-fold) (Lyons and Jacques, 2001; Whanger, 2004). Se is an essential component of antioxidant enzymes (e.g., glutathione peroxidases) that can decrease the risk of peroxidation of polyunsaturated fatty acids (PUFA) (Crespo et al., 1995; Demirel et al., 2004; Traulsen et al., 2004; Suzuki, 2005; Juniper et al., 2008). A positive correlation was observed between concentrations of unsaturated fatty acids (UFA) and the dietary content of Se (Crespo et al., 1995; Juniper et al., 2008). Our recent studies revealed that selenate (Se^{VI}) or selenite (Se^{IV}) changed the concentrations of fatty acids (FA) and conjugated linoleic acid (CLA) isomers, in particular in incubated ovine ruminal fluid (Wąsowska et al., 2006a,b), as well as in the liver, muscles and adipose tissues of experimental animals (Czauderna et al., 2004ab, 2007; Korniluk et al., 2006). On the other hand, microorganisms can reduce excessive doses of Se^{IV} or Se^{VI} to unabsorbable elemental Se or selenide forms. Moreover, ruminal bacteria are also able to synthesize Se-methionine (Se-Met) and Se-cysteine (Se-Cys), and then these Se-amino acids (Se-AA) are incorporated into microbial protein. The predominant Se-AA was Se-Cys when ruminal microbes were incubated with Se^{IV} or Se^{VI} (Whanger, 2004). The latter may represent a route by which a portion of inorganic Se supplements in diets finds its way into a form metabolizable by ruminants, thus the possibility of improving the healthfulness of ruminant meat and milk by increasing the concentration of Se-Cys (Driscoll and Coperland, 2003; Gladyshev et al., 2004; Navarro-Alarcon and Cabrera-Vique, 2008).

Many epidemiological and experimental studies have documented that some saturated fatty acids (e.g., C14:0, C16:0 or C18:0) possess atherogenic and thrombogenic properties, while CLA isomers and PUFAn-3 improved anti-inflammatory status, immune response and benefited the cardiovascular system

(Hwang, 2000; Wahle et al., 2004; Wijendran and Hayes, 2004; Korniluk et al., 2006; Czauderna et al., 2007). Numerous investigations have shown that dietary PUFA (e.g., linoleic or linolenic acid) is directly incorporated by rumen bacteria, isomerized to other geometric and positional isomers, metabolized into CLA isomers, biohydrogenated to *trans*-vaccenic acid and finally to stearic acid (Raes et al., 2004; Choi et al., 2005; Heird and Lapillonne, 2005; Buccioni et al., 2012).

Considering the above, it seems reasonable to study the influence of various inorganic forms of Se (as Se^{IV} and Se^{VI}) and different concentrations of Se^{IV} and Se^{VI} on the concentration of selected fatty acids, especially CLA isomers and *trans11*C18:1 (*t11*C18:1) in *in vitro* incubated ruminal fluids with linoleic acid (LA). LA added to incubated ruminal fluids is also an extra source of energy. Therefore, the major objective of the current study was to examine the hypothetical effect of Se^{IV} and Se^{VI} on biohydrogenation of LA in *in vitro* incubated ruminal fluid of sheep.

MATERIAL AND METHODS

Animals and diets

Eight ruminally fistulated mature sheep were fed 1 kg dry matter (DM)/d of a mixed diet comprising meadow hay, barley, molasses, soyabean meal, and a mixture of vitamins and minerals, at 500, 299.5, 100, 91, and 9.5 g/kg DM, respectively, fed in equal meals of 500 g at 8.00 and 16.00 h. Ruminal digesta samples were taken before feeding in the morning from each sheep and kept at 39°C, strained through linen cloth before use for *in vitro* incubation.

Chemicals

Linoleic acid (LA), fatty acid methyl ester (FAME) standards, sodium selenite (Se^{IV}) and sodium selenate (Se^{VI}) were from Sigma (Poole, Dorset, UK). *Cis9trans11*CLA (95-97%) and *trans10cis12*CLA (95-97%) were supplied by Larodan Fine Chemicals AB (Sweden).

Other reagents were of analytical grade. Water used for the preparation of mobile phases and chemical reagents was prepared using an Elix™ water purification system (Millipore).

Incubation with ruminal fluid in vitro

Strained ruminal fluids were incubated *in vitro* either alone or with a combination of LA and two concentrations of Se as Se^{IV} or Se^{VI} for the determination

of interactions in the metabolism of LA. All *in vitro* experiments were performed on four different days using samples withdrawn from eight different sheep.

One ml of strained ruminal fluid was added under CO_2 to Pyrex tubes (120 x 11 mm) containing one of the following:

- 0.2 ml of distilled water (the control incubated ruminal fluid): the negative control group (RF);
- 0.1 ml of water and 0.1 ml of water solution containing 2 μg Se as Se^{IV} or Se^{VI} (the high Se level in incubated fluid: 1.67 μg Se per ml of incubated fluid; $Se^{IV}H$ or $Se^{VI}H$, respectively): the positive control groups;
- 0.1 ml of water and 0.1 ml of water solution containing 0.2 μg Se as Se^{IV} or Se^{VI} (the low Se level in incubated fluid: 0.167 μg Se per ml of incubated fluid; $Se^{IV}L$ or $Se^{VI}L$, respectively): the positive control groups;
- 0.1 ml of water and 0.1 ml of water mixture containing 2 mg LA (1.67 mg LA per 1 ml of incubated fluid (LA)): the positive control group;
- 0.1 ml of water mixture containing 2 mg LA and 0.1 ml of water solution containing 2 μg Se as Se^{IV} or Se^{VI} (1.67 μg Se and 1.67 mg LA per ml of incubated fluid; $LASe^{IV}H$ or $LASe^{VI}H$, respectively): the experimental groups;
- 0.1 ml of water mixture containing 2 mg LA and 0.1 ml of water solution containing 0.2 μg Se as Se^{IV} or Se^{VI} (0.167 μg Se and 1.67 mg LA per ml of incubated fluid; $LASe^{IV}L$ or $LASe^{VI}L$, respectively): the experimental groups.

Thus, the final volumes of incubated *in vitro* ruminal fluid were always 1.2 ml. Tubes with examined ruminal fluid were incubated at 39°C. Tubes were removed after 0, 6, 12, 18, and 24 hrs of *in vitro* incubation, heated for 10 min in a block heater at 100°C and stored at -20°C before determination of fatty acid concentrations. Free fatty acids were extracted and analysed as described below. Samples of the original strained ruminal fluid were stored at -20°C for later protein analysis.

Fatty acid extraction and preparation of fatty acid methyl esters (FAME)

The methods of hydrolysis and derivatization were as described previously (Christie, 2003; Wasowska et al., 2006a;). Briefly, 1.2 ml of incubated *in vitro* ruminal samples were mixed with 1.25 ml of acidified salt solution (17 mM NaCl in 1 mM H_2SO_4). One hundred μl of 200 μg/ml C17:0 were added as an internal standard, followed by 2.5 ml of methanol. The mixture was vortexed for 1 min, then 2.5 ml of chloroform containing added 0.2 mg/ml butylated hydroxy-toluene (BHT) were added and the mixture was vortexed again, for 2 min. The upper layer was removed by aspiration. The lower layer was dried by passing through

anhydrous sodium sulphate and the solvent was evaporated in a centrifugal evaporator (Savant AES2010, Thermo Electron Corporation, Basingstoke, Hants., UK).

Derivatization of the extracted fatty acids to FAME was carried out using a procedure that contained a short, mild esterification step that minimized isomerization of CLA. The dried extract was re-suspended in 0.5 ml of toluene, the suspension was vortexed, then 1 ml of H_2SO_4/methanol (1%, v/v, conc. H_2SO_4 in methanol) was added. One hundred µl of 200 µg/ml C15:0 were added as a second internal standard to monitor recovery through the derivatization procedure. The tube was flushed with N_2 then closed with a glass stopper and incubated at 50°C for 1 h. The tube was cooled, opened, 2.5 ml of 5% NaCl were added, the tube was vortexed, then 1 ml of *iso*-hexane was added and the tube was vortexed again. When layers had formed, sometimes aided by brief centrifugation, the upper layer was transferred to a fresh tube and the *iso*-hexane extraction was repeated twice on the lower phase. The *iso*-hexane fractions were pooled and 1.5 ml of 2% $KHCO_3$ were added. The mixture was vortexed and allowed to settle, once again aided by brief centrifugation if necessary. The upper layer was removed, evaporated, and re-suspended in 0.2 ml of *iso*-hexane/BHT, then transferred to a GC vial.

Chromatographic equipment and FAME analysis

The gas chromatograph was an Agilent 6890 instrument (Agilent Technologies UK Ltd, Stockport, Cheshire, UK) equipped with a quadrupole mass selective detector (Model 5973N), flame-ionization detector, injection port, and a fused silica capillary column (100 m × 0.25 mm) coated with a 0.2 µm film of cyanopropyl polysiloxane (CP-SIL 88; Varian Analytical Instruments, Walton-on-Thames, Surrey, UK).

The FAME concentrations in a 1 µl of sample at a split ratio of 15:1 were determined using a temperature gradient programme. The temperature programme was as follows: 80°C for 1 min; 25°C/min to 160°C then held for 3 min; 1°C/min to 190°C then held for 5 min; 2°C/min to 230°C then held for 25 min. The carrier gas was helium and the column was operated at constant pressure (20 psi) with a flow rate of 0.5 ml/min. Injector and detector temperature was maintained at 250°C and 275°C, respectively.

Statistical analyses

Statistical analyses of the effects of LA, Se^{IV} and Se^{VI} on CLA isomers and other fatty acids in *in vitro* incubated ruminal fluids were conducted using the non-parametric Mann-Whitney U test. The data were presented as means.

RESULTS AND DISCUSSION

The influence of Se^{IV} on concentrations of CLA isomers and other fatty acids in in vitro incubated ruminal fluid with LA

Although factors altering ruminal fermentation and the microbial population are undoubtedly keys to controlling the regulation of biohydrogenation and CLA isomer synthesis, very few studies have directly associated production of CLA isomers and their precursors in ruminant feeds enriched in inorganic Se (Czauderna et al., 2004a,b). Therefore*, in vitro* studies were conducted to determine the effect of Se^{IV} and Se^{VI} on biohydrogenation of UFA and production of CLA isomers, *t11*C18:1 (TVA), and other geometrical and positional isomers of unsaturated fatty acids (UFA) in the ruminal fluid containing extra LA.

In the current study, ruminal fluid from eight sheep was incubated *in vitro* with extra LA and Se^{IV} or Se^{VI}; samples were removed for the analysis of free fatty acids at 6 h intervals. The influence of the low (0.167 μg Se/ml; L) and high (1.67 μg Se/ml; H) concentrations of Se^{IV} and Se^{VI} in the ruminal fluid on the CLA isomers and other FA compositions in assayed samples are shown in Table 1. The concentrations of all CLA isomers are diminutive (usually below detection limits) in all *in vitro* incubated ruminal fluid (RF) without and with only Se^{IV} or Se^{VI} at both levels (Se^{IV}L, Se^{IV}H, Se^{VI}L and Se^{VI}H). Addition of the lower and higher amount of Se^{IV} (Se^{IV}L or Se^{IV}H) to the ruminal fluid with LA numerically or statistically usually decreased the concentrations of individual CLA isomers, especially *cis9trans11*CLA (*c9t11*CLA), as well as the sum of all CLA isomers (ΣCLA) in *in vitro* incubated fluid with LA in comparison with the ruminal fluid containing only LA. Moreover, the higher level of Se^{IV} (Se^{IV}H) in the ruminal fluid with LA more effectively decreased the formation yield of ΣCLA isomers, especially *c9t11*CLA, than the lower of Se^{IV} (Se^{IV}L) in the ruminal fluid with LA incubated from 6 to 18 hrs. The addition of Se^{IV}H to the ruminal fluid with LA more effectively decreased the *c9t11*CLA/ (*c9t11*CLA+LA) ratios than the addition of Se^{IV}L to the fluid with LA, especially for the longer incubation time (i.e. >6 hrs). Thus, our current results documented that the higher concentration of Se^{IV} in the ruminal fluid (Se^{IV}H) more efficiently reduced the yield of isomerization of LA to *c9t11*CLA than the lower concentration of Se^{IV} (Se^{IV}L). Considering the above and the literature data (Buccioni et al., 2012), we argue that Se^{IV} in a dose - dependent manner decreased the yield of enzymatic isomerization by *cis12trans11*-isomerase, which turns the *cis12*-bond into a *trans11*-bond (i.e. formation of geometric and positional isomers). Interestingly, this bacterial isomerase has particularly specific requirements for free carboxylic groups (i.e., free unsaturated fatty acids), and especially groups with a free diene possessing the *cis9cis12*-geometry (e.g., LA, *c6c9c12*C18:3 or *c9c12c15*C18:3 (αLNA)). Therefore, Se^{IV} in dose - dependent

manner decreased the concentration of *c9t11*CLA in *in vitro* incubated ruminal fluid with LA (Table 1) compared with the fluid containing only LA. Similarly, Se^{IV} decreased the concentration of *c9c11*CLA in the incubated fluid with only LA; the decrease in the concentration of *c9c11*CLA is higher in the ruminal fluid containing LA and higher concentration of Se^{IV}. Moreover, the addition of Se^{IV} to the ruminal fluid containing LA reduced the formation yield of *t9t11*CLA compared with the incubated fluid with only LA. This effect of Se^{IV} on the concentration of *t9t11*CLA is similar for both concentrations of Se^{IV} in the ruminal fluid. Therefore, we argue that the maximal reduction of the formation yield of this *tt*CLA isomer *via* bacterial geometrical and positional isomerizations was already achieved at the lower concentration of Se^{IV} (Se^{IV}L) in the ruminal fluid with LA. Therefore, the higher concentration of Se^{IV} (Se^{IV}H) in the incubated fluid with LA had no effect on further increasing the formation yield of *t9t11*CLA. The addition of Se^{IV} to the incubated fluid with LA leads to a decrease in the concentration of *t10c12*CLA compared with the fluid containing only LA. Moreover, the accumulation of this CLA isomer decreased at 6 and 12 hrs when the higher concentration of Se^{IV} (Se^{IV}H) was present in the ruminal fluid with LA. Thus, our investigations documented that Se^{IV} reduced the capacity of bacterial isomerase, which turns the *cis9*-bond into a *trans10*-bond. As a consequence, Se^{IV} added to the fluid with LA decreased values of the ratio of *c9t11*CLA to *t10c12*CLA ($R_{cis9trans11/trans10cis12}$) from 0 to 12 hrs of *in vitro* incubation compared with the fluid containing only LA (Table 1).

The effect of both concentrations of Se^{IV} in the fluid with LA was opposite after longer incubation time (after 12 hrs); considering the above we argue that Se^{IV} during incubation of the ovine fluid is gradually oxidized by some ruminal microorganisms to Se^{VI} (selenate). Therefore, after 12 hrs of incubation of the ruminal fluid with LA and Se^{IV} (as Se^{IV}L or Se^{IV}H), the effect of Se^{IV} on the accumulation of *t10c12*CLA is similar to the effect of Se^{VI} added to the incubated fluid with LA. Considering all above results, we can argue that Se^{IV} reduced the activity of linoleic isomerase of ruminal bacteria (e.g., *B. fibrisolvents*), which isomerised *cis-9* and *cis-12* bonds of unsaturated fatty acids in *in vitro* incubated ruminal fluid containing LA. Hence, the lower concentrations of CLA isomers were found in the ruminal fluid with LA and Se^{IV} irrespectively of the concentration of Se^{IV}. As a consequence of the above, the higher concentration of Se^{IV} in the incubated fluid with LA was responsible for the higher concentration of LA from 12 hrs incubation than in the ruminal fluid with only LA (Table 1).

*Trans11*C18:1 (TVA) is the biohydrogenation intermediate of isomerised LA; this first step of biohydrogenation (i.e. the initial biohydrogenation; *i*BH) is the fast reaction catalyzed by microbial *reductase* of group A and B bacteria (Buccioni et al., 2012). As can be seen from results summarized in Table 1, the concentration of TVA increased throughout the incubations, especially in the ruminal in the

Table 1. Effects[1] of and two levels of Se^{IV} and Se^{VI} (low, L; high, H) on the concentration of selected fatty acids (μg/ml) in *in vitro* incubated ovine ruminal fluid containing linoleic acid (LA)

Group and *in vitro* incubation time, h		*c9c12*C18:2 (LA)	*t11*C18:1 (TVA)	*c9*C18:1	*c11*C18:1	*C20:5n-3*	TVA / *TVA+c9t11* (iBH_{index})[4]	*C18:0*	C18:0 / C18:0+TVA (fBH_{index})[5]	*CLA isomers*[2] *c9t11*	*t10c12*	*c9c11*	*t9t11*	∑CLA[6]	*c9t11* / *c9t11+LA* isomerase index[7]	$R_{c9t11/t10c12}$[8]
RF[3]	0	13^{A}	13^{a}	12^{a}	0.4^{a}	4.0^{a}	1^{a}	133^{a}	0.911^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-
$Se^{IV}L$	0	14^{A}	15^{a}	12^{a}	0^{a}	5.0^{a}	1^{a}	143^{a}	0.905^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-
$Se^{IV}H$	0	14^{A}	14^{a}	12^{a}	0.5^{a}	5.1^{a}	1^{a}	146^{a}	0.913^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-
$LASe^{IV}L$	0	1608^{B}	14^{a}	19^{b}	0^{a}	7.0^{a}	0.828^{b}	144^{a}	0.911^{a}	2.9^{b}	0.7^{b}	0^{a}	0.5^{a}	4.1^{b}	0.0018^{b}	4.14^{a}
$LASe^{IV}H$	0	1534^{B}	17^{a}	20^{b}	0^{a}	7.0^{a}	0.833^{b}	156^{a}	0.902^{a}	3.4^{b}	0.9^{b}	0.6^{b}	2.1^{b}	6.9^{b}	0.0022^{b}	3.78^{b}
$Se^{VI}L$	0	14^{A}	15^{a}	13^{a}	0^{a}	5.0^{a}	1^{a}	143^{a}	0.905^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-
$Se^{VI}H$	0	14^{A}	14^{s}	14^{a}	0^{a}	5.4^{a}	1^{a}	142^{a}	0.910^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-
$LASe^{VI}L$	0	1786^{B}	13^{a}	18^{a}	0^{a}	7.0^{a}	0.844^{b}	142^{a}	0.916^{a}	2.4^{b}	0^{a}	0^{a}	0^{a}	2.4^{b}	0.0013^{b}	-
$LASe^{VI}H$	0	1706^{B}	13^{a}	18^{a}	0^{a}	9.0^{a}	0.783^{c}	142^{a}	0.916^{a}	3.6^{b}	1.2^{a}	0^{a}	0.5^{a}	0^{a}	0.0021^{b}	3.00
LA	0	1721^{B}	14	19^{b}	0^{a}	9.0^{a}	0.778^{c}	145^{a}	0.912^{a}	4.0^{b}	1.5^{a}	0^{a}	0.5^{b}	6.0^{b}	0.0023^{b}	2.67^{ab}
RF	6	10^{A}	15^{a}	12^{a}	0^{a}	4.1^{a}	1^{A}	148^{a}	0.908^{a}	0^{A}	0^{a}	0^{a}	0^{a}	0^{A}	0^{A}	-
$Se^{IV}L$	6	10^{A}	14^{a}	13^{a}	0^{a}	5.2^{a}	1^{A}	150^{a}	0.915^{a}	0^{A}	0^{a}	0^{a}	0^{a}	0^{A}	0^{A}	-
$Se^{IV}H$	6	10^{A}	14^{a}	13^{a}	0^{a}	5.1^{a}	1^{A}	151^{a}	0.915^{a}	0^{A}	0^{a}	0^{a}	0^{a}	0^{A}	0^{A}	-
$LASe^{IV}L$	6	1232^{B}	33^{b}	31^{b}	0^{a}	7.0^{a}	0.122^{B}	138^{a}	0.807^{b}	238^{B}	26^{b}	14^{b}	32^{b}	309^{B}	0.162^{B}	9.15^{α}
$LASe^{IV}H$	6	1152^{B}	33^{b}	33^{b}	0^{a}	6.0^{a}	0.126^{B}	153^{a}	0.823^{b}	229^{B}	1^{b}	13^{b}	35^{b}	298^{B}	0.166^{B}	10.9^{a}
$Se^{VI}L$	6	10^{A}	14^{a}	13^{a}	0^{a}	4.6^{a}	1^{A}	149^{a}	0.914^{a}	0^{A}	0^{a}	0^{a}	0^{A}	0^{A}	0^{A}	-
$Se^{VI}H$	6	10^{A}	14^{a}	13^{a}	0^{a}	5.0^{a}	1^{A}	148^{a}	0.914^{a}	0^{A}	0^{a}	0^{a}	0^{A}	0^{A}	0^{A}	-
$LASe^{VI}L$	6	1259^{B}	42^{b}	32^{b}	0.5^{a}	8.0^{a}	0.130^{B}	140^{a}	0.769^{b}	281^{B}	31^{b}	16^{b}	43^{B}	370^{B}	0.182^{B}	9.07
$LASe^{VI}H$	6	1264^{B}	51^{c}	33^{b}	0^{a}	8.0^{a}	0.166^{B}	144^{a}	0.738^{b}	257^{B}	43^{b}	16^{b}	46^{B}	362^{B}	0.169^{B}	5.98
LA	6	1210^{B}	48^{a}	32^{b}	0^{a}	8.0^{a}	0.144^{B}	140^{a}	0.745^{b}	285^{B}	37^{b}	18^{b}	45^{B}	385^{B}	0.191^{B}	$7.70^{a\alpha}$
RF	12	4^{A}	20^{A}	12^{a}	0^{a}	4.4^{a}	1^{Aa}	165^{a}	0.892^{a}	0^{A}	0^{a}	0^{a}	0^{A}	0^{Aa}	0^{A}	-
$Se^{IV}L$	12	5^{A}	18^{A}	12^{a}	0^{a}	4.6^{a}	0.947^{bc}	167^{ab}	0.903^{a}	1^{A}	0^{a}	0^{a}	0.5^{A}	1.5^{b}	0.167^{B}	-
$Se^{IV}H$	12	5^{A}	19^{A}	12^{a}	0^{a}	4.9^{a}	0.965^{bc}	170^{ad}	0.899^{a}	0.7^{A}	0^{a}	0^{a}	0.6^{A}	1.3^{a}	0.123^{B}	-
$LASe^{IV}L$	12	1043^{B}	101^{B}	35^{b}	1.8^{b}	6.0^{a}	0.328^{Be}	130^{ac}	0.563^{c}	207^{B}	38^{b}	15^{b}	44^{B}	305^{Bc}	0.166^{B}	5.45
$LASe^{IV}H$	12	1084^{B}	94^{B}	34^{b}	0^{a}	9.0^{a}	0.379^{Be}	142^{ae}	0.602^{c}	154^{Bb}	26^{bd}	14^{b}	51^{B}	245^{Bc}	0.124^{β}	5.92^{α}
$Se^{VI}L$	12	5^{A}	19^{A}	12^{a}	0^{a}	4.9^{a}	1^{A}	164^{a}	0.896^{a}	0.7^{A}	0^{a}	0^{a}	0.5^{A}	1.2^{a}	0.123^{B}	-
$Se^{VI}H$	12	5^{A}	18^{A}	12^{a}	0^{a}	4.8^{a}	0.964^{bc}	165^{a}	0.902^{a}	0^{A}	0^{a}	0^{a}	0.5^{A}	0^{a}	0^{A}	-
$LASe^{VI}L$	12	1038^{B}	162^{C}	42^{b}	3^{b}	7.0^{a}	0.383^{Be}	140^{a}	0.464^{c}	260^{Bc}	61^{be}	22^{c}	72^{B}	415^{B}	0.200^{B}	4.26

Table 1. continued on the page 485

Table 1. continued

Group and *in vitro* incubation time, h		*c9c12*C18:2 (LA)	*t11*C18:1 (TVA)	*c9*C18:1	*c11*C18:1	*C20:5n-3*	TVA / *TVA+c9t11* (*i*BH$_{index}$)[4]	*C18:0*	C18:0 / C18:0+TVA (*f*BH$_{index}$)[5]	*CLA isomers*[2] *c9t11*	*t10c12*	*c9c11*	*t9t11*	∑CLA[6]	*c9t11* / *c9t11+LA* isomerase index[7]	R$_{c9t11/t10c12}$[8]
LASe^{VI}H	12	1078^{B}	170^{C}	30^{b}	1^{a}	8.0^{a}	0.389^{Be}	143^{a}	0.457^{d}	266^{Bc}	60^{be}	22^{bc}	67^{B}	415^{Bd}	0.198^{B}	4.43
LA	12	1022^{B}	198^{a}	43^{b}	4^{b}	6.0^{a}	0.463^{Be}	141^{a}	0.416^{d}	230^{B}	62^{be}	22^{bc}	70^{B}	384^{Bd}	0.184^{B}	3.71$^{\alpha}$
RF	18	2.3^{A}	19^{A}	10^{a}	0^{a}	4.1^{a}	1^{A}	170^{a}	0.899^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
Se^{IV}L	18	1.7^{A}	19^{A}	9^{a}	3^{a}	4.3^{a}	1^{A}	186^{ab}	0.907^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
Se^{IV}H	18	2.0^{A}	18^{A}	9^{a}	0^{a}	5.0^{a}	1^{A}	177^{ad}	0.908^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
LASe^{IV}L	18	788^{B}	334^{B}	48^{b}	8^{b}	6.0^{a}	0.669^{Ba}	141^{ac}	0.297^{b}	165^{B}	80^{B}	14^{b}	67^{B}	325^{Ba}	0.173^{B}	2.06
LASe^{IV}H	18	853^{B}	287^{Ba}	43^{b}	6^{b}	6.0^{a}	0.742^{Bb}	139^{ae}	0.326^{b}	100^{B}	53^{B}	13^{b}	52^{Ba}	217^{Bb}	0.105^{B}	1.89
Se^{VI}L	18	2.8^{A}	18^{A}	9^{a}	0^{a}	4.4^{a}	1^{A}	177^{af}	0.908^{a}	0^{A}	0^{A}	0^{a}	0.5^{A}	0.5^{A}	0^{A}	-
Se^{VI}H	18	2.5^{A}	21^{A}	10^{a}	0^{a}	4.7^{a}	1^{A}	167^{a}	0.888^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
LASe^{VI}L	18	819^{B}	448^{C}	36^{bc}	13^{b}	8.0^{b}	0.770^{Bb}	144^{ag}	0.243^{b}	134^{B}	67^{B}	15^{b}	79^{B}	295^{Bab}	0.141^{B}	2.00
LASe^{VI}H	18	834^{B}	375^{Bb}	51^{be}	10^{b}	7.0^{a}	0.691^{Bba}	142^{a}	0.275^{b}	168^{B}	71^{B}	18^{b}	87^{Bb}	344^{Ba}	0.168^{B}	2.37
LA	18	769^{B}	446^{Cc}	56^{be}	12^{b}	7.0^{a}	0.723^{Bba}	144^{a}	0.244^{b}	171^{B}	70^{B}	17^{b}	82^{Bb}	339^{Ba}	0.182^{B}	2.44
RF	24	0.6^{A}	18^{A}	8^{a}	0^{A}	4.4^{a}	1^{a}	172^{a}	0.905^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
Se^{IV}L	24	1.8^{A}	15^{A}	7^{a}	0^{A}	3.9^{a}	1^{a}	177^{a}	0.922^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
Se^{IV}H	24	0.1^{B}	18^{A}	7^{a}	0^{A}	4.8^{a}	1^{a}	187^{ab}	0.912^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
LASe^{IV}L	24	602^{C}	564^{Ba}	34^{b}	18^{B}	6.0^{a}	0.881^{b}	142^{a}	0.201^{b}	76^{Ba}	66^{B}	8^{b}	46^{B}	196^{Ba}	0.112^{B}	1.15
LASe^{IV}H	24	741^{C}	446^{Bb}	53^{b}	18^{B}	6.0^{a}	0.856^{b}	143^{ac}	0.243^{b}	75^{Ba}	77^{B}	8^{b}	57^{B}	217^{Ba}	0.092^{B}	0.97^{ab}
Se^{VI}L	24	0^{B}	17^{A}	9^{a}	1^{A}	4.6^{a}	1^{a}	179^{a}	0.913^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
Se^{VI}H	24	1.0^{A}	20^{A}	26^{bc}	24^{Ba}	4.6^{a}	1^{a}	185^{ad}	0.902^{a}	0^{A}	0^{A}	0^{a}	0^{A}	0^{A}	0^{A}	-
LASe^{VI}L	24	677^{C}	552^{B}	51^{b}	11^{Ba}	7.0^{a}	0.820^{b}	145^{a}	0.208^{b}	121^{Bb}	85^{B}	14^{b}	94^{B}	315^{Bb}	0.152^{B}	1.42
LASe^{VI}H	24	737^{C}	491^{Bc}	53^{b}	21^{B}	8.0^{b}	0.788^{b}	146^{ae}	0.229^{b}	132^{Bb}	81^{B}	19^{b}	99^{B}	332^{Bb}	0.152^{B}	1.63^{b}
LA	24	634^{C}	618^{Bd}	56^{bc}	24^{Ba}	7.5^{a}	0.879^{b}	144^{a}	0.189^{b}	85^{d}	55^{e}	22^{a}	79^{B}	241^{Bab}	0.118^{B}	1.54^{a}

[1] means in columns at the same incubation time with the different letter are significantly different at ^{a,b} P<0.05 or at ^{A,B} P<0.01, while differences at $^{\alpha,\beta}$P=0.1 are indicated as tendencies; [2] *t, c* – abbreviations for the geometrical forms: *trans, cis,* respectively; [3] RF (the reference fluid) - *in vitro* incubated 1ml of ruminal fluid with 0.2 ml of water (the control ruminal fluid); [4] the index of initial biohydrogenation (*i*BH$_{index}$) of *c9t11*CLA to *t11*C18:1 (the fast step of biohydrogenation (Buccioni et al., 2012); [5] the index of final biohydrogenation (*f*BH$_{index}$) of TVA to C18:0; [6] the concentration sum of CLA isomers: *c9t11*CLA, *t10c12*CLA, *c9c11*CLA and *t9t11*CLA; [7] the isomerase$_{index}$ - the index of *c9t11*CLA formation *via* bacterial isomerization; [8] R$_{c9t11/t10c12}$ - the concentration ratio of *c9t11*CLA to *t10c12* CLA (i.e., R = *c9t11*CLA/*t10c12* CLA)

ruminal fluid with LA regardless of the presence of Se^{IV} or Se^{VI}. Detailed analysis of results documented that, the addition of Se^{IV} to the ruminal fluid with LA decreased the concentration of *t11*C18:1 (TVA) compared with the fluid with only LA; a decrease in the loss of TVA was observed with increasing concentrations of Se^{IV}. Moreover, the presence of Se^{IV} in the fluid with LA stimulated the final biohydrogenation of TVA to C18:0 (the step limiting the rate of biohydrogenation of unsaturated fatty acids) (Buccioni et al., 2012). Therefore, the addition of Se^{IV} to the fluid with LA results in an increase in the values of the final biohydrogenation index (fBH_{index}) of TVA (Table 1). The values of fBH_{index} increased as the concentration of Se^{IV} in the ruminal fluid with LA increased. Thus, the current studies documented that Se^{IV} in a dose-dependent mannr stimulated reductase activity. Indeed, the values of the initial biohydrogenation index (iBH_{index}) of *c9t11*CLA to TVA (the fast step of biohydrogenation) was lower in the ruminal fluid containing simultaneously LA and Se^{IV} than in the fluid with only LA, as Se^{IV} added to the fluid with LA in a dose-dependent manner increased the yield of the final biohydrogenation of TVA (the product of the initial biohydrogenation) to C18:0 (Table 1).

According to the above, our detailed investigations also demonstrated that the addition of Se^{IV} to the fluid with LA leads to a decrease in the concentration of *c9*C18:1 and *c11*C18:1 compared with the fluid with only LA. The lower or higher amount of Se^{IV} added to the ruminal fluid resulted in an increase in the concentration of C18:0 from 6 hrs incubation compared with the control fluid (RF) and the fluid containing LA and Se as Se^{IV} (irrespectively of its concentration). Thus, the present study documented that Se^{IV} stimulated the the final biohydrogenation of unsaturated fatty acids (UFA) to C18:0 in the incubated ruminal-fluid. As can be seen from data summarized in Table 1, the amount of Se^{IV} added to the fluid had a negligible influence on the yield of the final biohydrogenation of UFA to C18:0. Thus, the evidence from the present studies documented that the lower concentration of Se^{IV} resulted in the maximal increase of the yield of the final biohydrogenation. On the other hand, the addition of LA to the ruminal-fluid with Se^{IV} reduced the yield of the final biohydrogenation of UFA; so, this yield is lower than the yield of the final biohydrogenation in the control ruminal fluid and fluid containing Se^{IV}.

Detailed analysis of the results indicated that the concentration of eicosapentaenoic acid (C20:5n3) wasis practically unchangedable throughout the incubation of the control ruminal fluid. The addition of Se^{IV} to the incubated fluid usually stimulated the accumulation of C20:5n-3; the increase in the concentration of C20:5n-3 was observed with increasing the concentration of SeIV in the ruminal fluid. The addition of LA to the incubated fluid, irrespectively of the presence of Se^{IV}, increased the concentration of C20:5n-3; this finding suggests that LA decreased the yield of biohydrogenation of C20:5n-3 in the incubated ruminal fluid.

The influence of Se^{VI} on the concentration of CLA isomers and other fatty acids in in vitro incubated ruminal fluid with LA

To analyse the differential effects of various forms of inorganic Se on the metabolism of LA, CLA isomer formation, and rate of disappearance in the ruminal fluid, Se^{VI} was incubated *in vitro* in the fluid with LA (Table 1). The effect of low (L) and high (H) levels of Se^{VI} in the fluid with LA on the concentration of CLA isomers differs from influence of Se^{IV} in the fluid with LA. Indeed, Se^{VI} in the ruminal fluid with LA usually more efficiently increased the concentration of *c9t11*CLA, *t10c12*CLA, *c9c11*CLA, and *t9t11*CLA from 6 until 24 hrs of incubation compared with the fluid containing LA, regardless of the presence of Se^{IV}. This finding documented that the addition of Se^{VI} to the incubated fluid more effectively stimulated *linoleic isomerase* activity than Se^{IV} in the ruminal fluid with LA. As a consequence, the addition of Se^{VI} to the ruminal fluid with LA usually resulted in an increase in the values of the isomerase index compared with addition of Se^{IV} to the incubated fluid with LA. Moreover, Se^{VI} added to the fluid containing LA usually more efficiently increased the concentration of *t10c12*CLA compared with the fluid with Se^{IV} and LA. Se^{VI} added to the fluid containing LA reduced the values of the concentration ratio of *c9t11*CLA to *t10c12*CLA ($R_{c9t11/t10c12}$) in fluid incubated for 12 hrs compared with the ruminal fluid with Se^{IV} and LA. These results can be explained by progressive reduction of added Se^{VI} by rumen bacteria in *in vitro* incubated ruminal fluid of sheep.

As can be seen from the results summarized in Table 1, Se^{VI}, like Se^{IV}, did not contribute to the accumulation of CLA isomers in the ruminal fluid without LA; the concentrations of all CLA isomers in these fluids are below the limit of detection (L_D). The maximal concentration of *c9t11*CLA was found at 6 hrs of incubation of the ruminal fluid with LA, irrespectively of the presence of Se^{VI} or Se^{IV}. A decrease was then observedin the concentration of *c9t11*CLA in these fluids as incubation time increased. Indeed, the concentration of *c9t11*CLA is affected by the biohydrogenation of this CLA isomer to TVA (Buccioni et al., 2012); this is the fast reaction catalysed by bacterial *reductase*. Therefore, the concentration of TVA (the product of the fast reaction) increased with increasing incubation time; the concentration of TVA in the fluid containing Se^{VI} and LA is higher than in the ruminal fluid with Se^{IV} and LA. Considering the above, we argue that Se^{VI} added to the fluid with LA more effectively increased reductase capacity, consequently, the yield of initial biohydrogenation (*i*BH) in comparison with Se^{IV} added to the ruminal fluid with LA. The highest accumulation of TVA was observed at 24 hrs of incubation of the ruminal fluid containing only LA. This finding documented that the highest activity of bacterial isomerase and, so, the yield of *i*BH, was observed in the incubated ruminal fluid containing only LA.

Moreover, the addition of Se^{VI} to the fluid increased the concentration of C18:0 (the final product of rate-determining biohydrogenation; *f*BH). As a consequence, Se^{VI} added to the ruminal fluid increased the value of the fBH_{index} compared with values of this index fBH_{index} in the incubated fluid with LA, irrespective of the presence of Se^{VI}.

Se^{VI} added to the ruminal fluid with LA decreased the metabolism yield of LA; in addition, the loss of LA in the incubated fluid decreased as the concentration of Se^{VI} in the fluid with LA increased. From the data shown in Table 1 it follows that both levels of Se^{VI}, in general, have a only minor and non-consistent influence on the concentrations *c9*C18:1, *c11*C18:1 and C20:5n-3 in the incubated ruminal fluid with LA.

The effect of ruminal microorganisms on isomerisation and biohydrogenation of unsaturated fatty acids in in vitro incubated fluids with Se^{IV} or Se^{VI}

The formation of CLA isomers and their precursors in ruminal fluids of sheep can be explained by the conversion of dietary LA, through group A ruminal bacteria (Bauman et al., 2003; Sieber et al., 2004; Buccioni et al., 2012). As the first step *cis,trans* and/or *trans,cis* conjugated fatty acids derived from LA, γ-linolenic acid (*c6c9c12*C18:3) or α-LNA (e.g.: *c9t11*CLA, *c6c9t11*C18:3 or *c9t11c15*C18:3) were formed due to the activity of an isomerase (linoleate isomerase; EC 5.2.1.5) from anaerobic bacterium *Butyrivibrio fibrisolvens*. In addition, low-fibre diets stimulated the capacity of other ruminal bacteria such as the *Megasphaera (n.) elsdenii strains* YJ-4 and T81 that are able to produce significant amounts of *t10c12*CLA (the other isomerization product of LA or biohydrogenated α-LNA), but but not some other strains (e.g., strains B159, AW106 and JL1) (Kim et al., 2002, Sieber et al., 2004; Buccioni et al., 2012). The results in our study show that the addition of two levels of Se^{IV} to the incubated ruminal fluid with LA slowed the rate of the isomerisation reaction of LA, therefore, the concentration of the main isomerisation product, *c9t11*CLA, and other minor isomers (like *t10c12*CLA), as well as the concentration of the sum of all CLA isomers are lower in *in vitro* incubated fluid with LA and the inhibition of isomerisation stimulated the increase of Se^{IV} in the ruminal fluid. Consequently, longer *in vitro* incubation of the ruminal fluid with LA in the presence of Se^{IV} resulted in the increase of the LA in fluid in comparison with the fluid with only LA. Moreover, the increase of the Se^{IV} concentration leads to a lower rate of disappearance of LA in the incubated ruminal fluid.

The second intermediates of the initial biohydrogenation of LA, *c6c9c12*C18:3 and α-LNA in ruminal fluid are formed as a result of hydrogenation of transitory intermediates (i.e. *c9t11*CLA, *t10c12*CLA, *c6c9t11*C18:3 or *c9t11c15*C18:3); these

second intermediates are: *t11*C18:1 (TVA) or *t10*C18:1 (predominantly formed in lower ruminal fluid pH). TVA or/and *t10*C18:1 accumulate in the incubated ruminal fluid as the final biohydrogenation of these second intermediates to stearic acid (C18:0) is the rate-determining step (Buccioni et al., 2012). This final biohydrogenation to C18:0 is effected by ruminal group B bacteria and the rate of this final biohydrogenation is lower compared with the rate of the initial biohydrogenation of transitory intermediates (Bauman et al., 2003; Buccioni et al., 2012). As can be seen from our results, the slowed rate of the isomerisation reaction of LA in ruminal fluids with LA and Se^{IV} resulted in a numerical decrease in the yield of the biohydrogenation of the transitory intermediate in *in vitro* incubated fluid and the decrease of TVA accumulation in the fluid is more evident with the higher concentration of Se^{IV} in the fluid. The current study clearly showed that the final biohydrogenation of the initial intermediate (i.e., TVA) occurred less rapidly, and therefore the reaction of the final biohydrogenation by bacteria B limited the accumulation of stearic acid (C18:0) in the incubated ruminal fluid.

Our results (Table 1) support the earlier observations of Kim et al. (2000) and Buccioni et al. (2012) that LA inhibited the growth of ruminal bacteria, and, e.g. at 1800 μM LA the growth of *B. fibrisolvens* A38 was completely inhibited and no CLA isomers as well as the final product (i.e. C18:0) of LA biohydrogenation was found (Kim et al., 2000; Sieber et al., 2004). Our results are consistent with the observation of Kim et al. (2000), therefore, the concentration of C18:0 was lower in *in vitro* incubated ruminal fluid with LA regardless of the presence of Se^{IV} or Se^{VI}. Interestingly, the addition of Se^{IV} or Se^{VI} to the ruminal fluid usually resulted in an increase in the concentration of C18:0 as well as *c9*C18:1 and C20:5n-3 compared with *in vitro* incubated control ruminal fluid (RF). Therefore, we could hypothesize that both chemical form of Se (i.e. Se^{IV} or Se^{VI}), irrespective of their concentrations, are not toxic towards growth of ruminal bacteria in *in vitro* incubated fluid.

As can be seen from the results summarized in Table 1, the effects of Se in the incubated ruminal fluid with LA depend critically on the oxidation state of the added Se-compound. Detailed analysis of our results revealed that the addition of Se^{VI} to the ruminal fluid with LA resulted in a more complex influence on LA metabolism in the ruminal fluid. The results in Table 1 indicate that the addition of Se^{VI} to the ruminal fluid with LA resulted in a decrease of isomerase capacity, as well as inhibited initial biohydrogenation of *c9t11*CLA, *t10c12*CLA, *c9c11*CLA, and *t9t11*CLA. These surprising results can be explained by formation of selenite (Se^{IV}) due to reduction of added Se^{VI} (selenate) by ruthe minal bacteria. Therefore, we could hypothesize that the formed Se^{IV} decreases bacterial *isomerase* capacity, while non-reduced selenate (i.e. remainder Se^{VI}) in the incubated fluid with LA decreased the yield of biohydrogenation of *c9t11*CLA, *t10c12*CLA and *t9t11*CLA.

Therefore, the addition of Se^{VI} to the ruminal fluid with LA resulted in a decrease in the rate of disappearance of LA and in the rate of TVA formation in the incubated fluid. We suggest that Se^{VI} is preferentially biohydrogenated by ruminal bacteria and the reduction of Se^{VI} to Se^{IV} competes with the reduction of double bonds of CLA isomers.

CONCLUSIONS

In conclusion, selenate (Se^{VI}) elevated the concentration of CLA isomers and the precursor of *c9t11CLA* in incubated ruminal fluid with linoleic acid (LA), therefore, we could hypothesise that feeding this chemical form of selenium with addition of free LA will improve the nutritive value of products derived from ruminants. In particular, meat, milk and dairy products should contain a higher concentration of CLA isomers derived directly from ruminal digesta, as well as from endogenous synthesis of conjugated dienes from *t11*C18:1 or *t7*C18:1. Moreover, our recent studies documented that feeding a diet enriched in selenate resulted in a substantial increase of other health-promoting-components like Se and Zn (essential elements) in the liver and muscles of sheep. Therefore, this is another possibility of improving the healthfulnss of ruminant meat and milk by increasing the concentration of Se-cysteine (an essential component of 22 seleno-proteins like glutathione peroxidase) and consequently by protecting PUFA from per-oxidation damage.

Further studies are required to clarify the effects of other selenium-compounds and fatty acids or vegetable oils on the profile of fatty acids, especially CLA isomers in ruminal fluid and to optimize the doses to be used.

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