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Preliminary study: From biofortified maize to cow milk enriched with selenium: An on-farm strategy using selenium-enriched silage

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Abstract: Selenium (Se) supplementation is a common practice in dairy nutrition. However, the use of biofortified feedstuffs remains a not fully realized strategy to enhance the Se content of animal derived products. This study explored an on-farm biofortification approach by incorporating Se-enriched maize silage into the total mixed ration (TMR) of dairy cows. Sixty Holstein cows were divided into a control group (CON), receiving a conventional diet with selenite supplementation (0.6 mg/kg Se in TMR), and an experimental group (EXP), in which conventional silage was replaced with high-Se silage (0.9 mg/kg Se in TMR). The trial lasted 22 weeks, including one week of adaptation and four weeks after supplementation, when Se concentrations in milk, Se transfer efficiency, and key milk components critical for the production of Se-enriched dairy products were assessed. The higher Se concentration in the TMR had no adverse effects on milk composition or antioxidant status. Milk Se concentration in the EXP group increased rapidly, reaching 68 µg/l within two weeks, significantly higher ($P < 0.005$) than in the CON group (27 µg/l). Se transfer efficiency to milk was also higher in the EXP group (13.9%) compared to the CON group (8.8%). The diverse Se species in biofortified silage, confirmed through the speciation analysis, may have contributed to these outcomes. However, the gradual decline in milk Se after the initial peak warrants further investigation into physiological factors or changes in silage Se speciation during storage.

Keywords: antioxidant status; dairy cows; selenium-enriched milk; selenium speciation; selenium supplementation

Selenium (Se) is widely recognised by dairy nutritionists as a vital trace element essential for the health and performance of cows (Duplessis et al. 2023). A selenium content of 0.3 mg/kg dry mat-

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ter (DM) in the total feed ration is generally considered adequate for lactating cows (NRC 2001). Despite the NRC's dietary recommendations being derived from extensive long-term empirical research, ambiguity persists regarding the precise definition of Se adequacy. This uncertainty extends to the reliability of Se biomarkers, cow health, previous vitamin E and Se status, basal diet composition, chemical form of Se in supplements, expected daily milk yield, dairy cow management, and the presence of stressors (Gerloff 1992; Salles et al. 2017). Optimising the feed composition, especially the forage choice, and reducing the costs are essential for improving milk production and quality (Codl et al. 2023), cows health and reproductive abilities (Kasna et al. 2023), and total dairy production efficiency (Wang et al. 2024). Commercial dairy farms commonly rely on external Se supplementation, including selenite (Se^{IV}), selenate (Se^{VI}), and seleno-yeast (SY), to maintain an adequate Se status in their herds. Notably, SY has been shown to be more efficient in this regard compared to Se^{IV} and Se^{VI} (Knowles et al. 1999; Ortman and Pehrson 1999; Gong et al. 2014). The supplementation strategy can also lead to the production of Se-enriched milk, offering an opportunity to develop marketable functional foods aimed at addressing low Se intake in the human population (Phipps et al. 2008; Doyle et al. 2011). However, various countries have imposed regulations on the maximum permitted Se supplementation for cattle. For instance, the European Union (EU) stipulates a limit of 0.568 mg/kg Se in DM for the total diet (EU 2013). Conversely, discussions regarding the need to revise ruminant nutrition recommendations to values slightly above the current nutritional requirements are present in the literature (Salles et al. 2017). This concept is supported by numerous experiments investigating basal diets supplemented with Se at levels permitted within the EU or slightly above (Phipps et al. 2008; Gong et al. 2014; Barbe et al. 2020). Moreover, an emerging strategy combines inorganic Se salts with SY in TMR, providing robust milk fortification while reducing the costs for SY (Azorin et al. 2020; Barbe et al. 2020).

In this study, we adopted an unconventional approach by feeding Se-biofortified maize silage, inspired by Seboussi et al. (2016), who found that Se-enriched silage and SY were similarly effective in fortifying cow's milk and were economically comparable sources of Se for cows. This study aimed to explore the potential of Se-enriched fodder, produced by ensiling maize treated with foliar selenate spray in the field, to enhance Se content in dairy milk. Specifically, the objectives were: *i*) to assess the feasibility of further enhancing the Se levels in milk by substituting Se-enriched silage for conventional low-Se silage in cows already receiving a standard level of Se supplementation; *ii*) to evaluate the temporal dynamics of Se concentration in milk, the efficiency of dietary Se transfer to milk, and major milk components during the supplementation trial; and *iii*) to verify that the combined use of Se-enriched silage and existing inorganic Se supplementation does not adversely affect cow performance, blood-based enzymatic activities, or antioxidant status.

MATERIAL AND METHODS

Experimental design

The study was conducted in accordance with Czech legislation for the protection of animals against cruelty (Act No. 246/1992) and with Directive 2010/63/EU on the protection of animals used for scientific purposes. This type of research does not compromise animal welfare, as the procedures implemented neither induced stress nor caused pain, and no additional handling or intervention beyond routine farm management practices was required.

The supplementation trial was conducted on a dairy farm located in Central Bohemia, Czech Republic (GPS: 49.8347N, 15.2558E). The farm manages a herd of 700 Holstein dairy cows that are milked in a herringbone parlour three times daily. For this study, 60 cows were selected to form a relatively uniform group based on calving dates (December 6, 2022, to January 16, 2023) and parity (2^{nd} and 3^{rd}), thereby standardising the stage of lactation across the cohort. The cows were randomly assigned to either the control group (CON; $n = 30$) or the experimental group (EXP; $n = 30$) and were housed in a free-stall system with a concrete base and bedding composed of separated manure solids. The trial was initiated with a one-week pre-supplementation period during which both groups received the same total mixed ration (TMR), fortified with a commercial vitamin-mineral premix (Table 1).

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Table 1. Composition of total mixed ration (TMR) for control (CON) and experimental (EXP) cows in a 22-week supplementation trial, including relative proportions of individual feed ingredients on a fresh matter basis (total daily fresh weight: 54.2 kg) and corresponding selenium intake data

Ingredient	Relative proportion (%)	
Maize silage	46.13	
Clover grass haylage	20.30	
Wet distillers grains with solubles	3.69	
Barley straw	0.55	
Malt flour	1.29	
Maize-cob mix	4.80	
Molasses	1.11	
Wheat	6.31	
Barley	2.44	
Soybean extruded meal	1.44	
Rapeseed extruded meal	6.64	
Maize	1.77	
Extruded rapeseed	1.99	
Urea milk	0.46	
Mineral feed additive – lactation[1]	0.66	
Limestone	0.13	
Sodium chloride	0.07	
Lithothamne	0.22	
Parameter	CON	EXP
Daily dry matter intake (kg)	27.3	27.9
Se content in TMR (mg/kg, DM)	0.58	0.89
Se daily intake (mg per cow)	15.8	24.8

[1]Commercial premix contained 180 g/kg Ca, 90 g/kg Na, 70 g/kg Mg, 40 g/kg P, 7 000 mg/kg Zn, 3 500 mg/kg Mn, 1 200 mg/kg Cu, 150 mg/kg I, 50 mg/kg Co, and 40 mg/kg Se (as selenite)
DM = dry matter

Water (a nutritionally irrelevant source of Se) was provided *ad libitum*, and TMR was delivered twice daily, with several additional replenishments throughout the day, ensuring an intake of 54 kg of fresh matter per cow per day. Beginning February 22, 2023, the diet of the EXP group was additionally supplemented with Se by replacing the entire portion of conventional maize silage with an equivalent mass of Se-enriched maize silage, while maintaining the selenite supplementation. On June 14, 2023, the diet of the EXP group was reverted to the basal diet administered to the CON group, initiating a one-month post-supplementation period.

Maize biofortification

Maize (*Zea mays* cv. RGT Attraxxion) is cultivated on-farm to ensure sufficient silage availability to the dairy herd. To provide an additional source of Se in the experimental TMR, the foliar treatment of a randomly selected 17-hectare field of maize was used with sodium selenate (Na_2SeO_4) solution at a Se dose of 40 g/ha during the BBCH 61 growth stage using a Tecnoma Laser 4240 self-propelled sprayer. Maize for silage was harvested at the approximately BBCH 85 stage (early dough stage) using a John Deere 7350 forage harvester. The plant material (yielding 29.3 t/ha at 35% DM) was shredded to a particle size of 10 mm and ensiled separately for Se-enriched and control maize in silage bags (three-layer polyethylene foil) for six months. A silage additive (Bonsilage SPEED M, Schaumann, Czech Republic) was applied during ensiling. After this period, the silage bags were opened and progressively unloaded to feed the dairy herds.

Sampling management

During the 22-week experimental period, whole blood (WB) and milk samples were collected on 13 shared sampling days, TMR samples on nine days, and maize silage on four days, with all sampling days evenly distributed throughout the study. The samples collected on the first two sampling days, February 15th and 22nd, are denoted as wk(–1) and wk(0), respectively, representing the pre-supplementation period. Eight cows from each treatment group were randomly selected on each sampling day for WB (two aliquots per cow, 2 × 8 ml) and milk (two aliquots per cow, 2 × 25 ml) collection. Whole blood was drawn from the coccygeal vein using a standardised sampling device (HEMOS H02; GAMEDIUM, Czech Republic), with veterinary assistance. The WB samples were transferred to heparinised 9-ml tubes (VACUETTE; Greiner BIO-ONE, Austria) and stored at 4 °C. The first WB aliquot was centrifuged (Universal 320 R, Hettich, Germany) at 2 000 × *g* (4 °C) for 15 min, not later than 2 h post-collection, to separate plasma and obtain red blood cells (RBC). The plasma and RBC samples were stored at –60 °C and subsequently shipped on dry ice to a laboratory for the determination of enzymatic activities and antioxidant status markers, where they were stored

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at –60 °C until analysis. The second WB aliquot was stored at –20 °C until determination of total Se (Se_{tot}) by inductively coupled plasma mass spectrometry (ICP-MS). Both milk aliquots were immediately refrigerated at 4 °C post-milking. The first aliquot (M1) was further divided into two subsamples (M1A and M1B). M1A was used for on-farm somatic cell count (SCC) analysis ($\times 10^3$ cells/ml) using a cell counter (DeLaval, Tumba, Sweden). M1B, along with the second aliquot (M2), was transported to a laboratory in a cooling box for Se_{tot} concentration analysis (ICP-MS) and for assessment of milk components and basic parameters, respectively. TMR samples were collected directly from a trough, while maize silage samples were taken during silage bag unloading. Both the TMR and silage samples were treated as composite samples, created by combining multiple samples collected on the same day. These composites were mixed, reduced to approximately 50 g, and placed in a 50-ml test tube for storage at –20 °C, pending determination of Se_{tot} content and Se chemical species.

Laboratory analysis

The compositional characteristics of milk, including fat, protein, casein, lactose, citric acid, and total solids (all given as % of the milk fresh weight), were analysed using a Fourier-transform infrared milk analyser (MilkoScan FT 120; Foss Electric A/S, Denmark). Blood parameters, specifically the activities of glutathione peroxidase (GPx; Flohe and Gunzler 1984), catalase (CAT; Goth 1991), superoxide dismutase (SOD; Marklund and Marklund 1974), total antioxidant capacity (TAC; Erel 2004), and total oxidant status (TOS; Erel 2005), were measured in RBC lysates. The lysates were prepared by vortexing 100 µl of RBC with 900 µl of cold water, followed by incubation at 4 °C for 15 min and subsequent centrifugation at 10 000 × *g* for 10 minutes. Additionally, glutathione reductase (GR; Cribb et al. 1989) activity was measured in plasma. All blood assays were adapted for a UV-Vis microplate reader (Reader Synergy H1, Biotek Instruments, USA) using 96-well plates. Silage and TMR samples were dried in an oven at 55 °C, then homogenised using a 1-mm mesh grinder. A 400-mg portion of the dried biomass was accurately weighed (±0.1 mg) and digested with a mixture of 8 ml HNO_3 (Analpure®; Analytika, Czech Republic) and 2 ml H_2O_2 (Rotipuran®, Carl Roth, Germany) at 195 °C for 30 min using a closed-vessel microwave system (Ethos One, Milestone, Germany). Similarly, 2-ml aliquots of milk were digested with a mixture of 6 ml HNO_3 and 2 ml H_2O_2 under the same conditions. Aliquots of WB (250 µl) were weighed into 15-ml polypropylene tubes and diluted 50-fold with a diluent containing 0.01% (v/v) Triton® X-100 (extra pure; Carl Roth, Germany). Selenium concentration was quantified using an ICP-MS (Agilent 8900; Agilent Technologies Inc., USA) operated in hydrogen mode. For quality assurance, certified reference materials Peach leaves (SRM-1547, NIST) and Bovine liver (BCR®-185R), and procedural blanks were analysed in parallel. An ion-pairing reversed-phase (IP-RP) high-performance liquid chromatography (HPLC; Agilent 1260, Agilent Technologies Inc., USA) coupled with ICP-MS was employed to quantify Se species in enzymatic hydrolysates of maize silage. The sample preparation and analytical procedures followed the methodology outlined by Mrstina et al. (2024).

Data processing and statistics

Data on whole blood, milk, enzymatic activity, antioxidant parameters, milk composition, and animal performance were analysed using SPSS statistical software (v29.0; IBM Corporation, USA). The Mann-Whitney *U* test, a non-parametric alternative for comparing the median of two independent groups, was used to assess differences between the CON and EXP groups at individual sampling weeks. Statistical significance was set at $P < 0.05$. Throughout the study, results are presented as means ± standard deviation (SD), with analyte contents in solid matrices expressed on a dry matter (DM) basis unless otherwise specified. The transfer efficiency of dietary Se to milk was calculated as the ratio of daily Se intake from the TMR to the amount of Se excreted in milk per cow on the sampling day.

RESULTS

Selenium analysis in silage and TMR

Silage contained 0.95 ± 0.14 mg/kg Se_{tot} in the Se-enriched variant and 0.03 ± 0.01 mg/kg in the control. Speciation analysis of Se in both Se-enriched

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chopped maize and silage was performed to confirm the transformation of the selenate spray into organic selenium (Se_{org}) forms within the maize. The major Se species in the representative enzymatic hydrolysate of chopped maize was selenomethionine (SeMet; 0.53 mg/kg Se; 53% of Se_{tot}), while inorganic species were negligible (<0.01 mg/kg Se^{IV} and 0.03 mg/kg Se^{VI}). However, the accurate speciation analysis in the silage was limited by a low method recovery of Se (19%), with column recovery at 27%. Despite this, the low levels of non-proteinaceous Se^{IV} (0.01 mg/kg) and Se^{VI} (0.03 mg/kg) can be considered consistent, in contrast to the suspiciously low content of SeMet (0.13 mg/kg Se). The analysis of Se_{tot} in TMR revealed 0.58 ± 0.03 mg/kg Se in DM of the diet for CON animals and 0.89 ± 0.07 mg/kg Se for EXP animals.

Selenium in whole blood

The concentrations of Se in WB over the 22-week trial, influenced by Se_{tot} and the dietary Se source in the TMR fed to cows, are shown in Figure 1A. The CON group, supplemented primarily with Se^{IV} provided through a mineral-vitamin premix, exhibited stable Se concentrations in WB, averaging 242 ± 18 µg/l throughout the trial. A slight but progressive increase in Se concentrations was observed, peaking at 262 ± 20 µg/l in wk(12). Subsequently, Se concentrations slightly declined (247 ± 19 µg/l) in wk(16) and wk(20), aligning with the overall mean of the CON dataset. The EXP group, which received Se from both the mineral premix and the Se-enriched silage, showed similar Se levels to the CON group until wk(1). Differences between the groups emerged during wk(2–4), though they were not statistically significant ($P > 0.18$). From wk(5) onwards, the differences became significant ($P < 0.05$). Selenium concentrations in WB plateaued at 301 ± 15 µg/l during wk(10–12), with a minor decrease observed in wk(16) and wk(20), despite the EXP herd transitioning back to low-Se silage in wk(16).

Selenium in milk

The time-resolved Se concentration in milk, in response to Se_{tot} and dietary Se source in TMR, is shown in Figure 1B. The CON group exhibited a stable Se concentration in milk, averaging 27 ± 4 µg/l throughout the trial. After the preliminary adaptation period in wk(–1) and wk(0), a significant difference ($P < 0.005$) emerged between CON and EXP milk samples in wk(1). The highest Se concentrations (68 ± 7 µg/l) were observed in wk(2–3), followed by a gradual decrease toward the end of the supplementation period in wk(16), while maintaining a significant difference ($P < 0.005$) between the EXP and CON groups. By wk(20), four weeks after ending the supplementation with biofortified silage, Se levels in milk had dropped to 31 ± 4 µg/l. Notably, two local minima in milk Se concentration appeared in wk(5) and wk(12) in EXP cows, mirroring similar trends in the CON group.

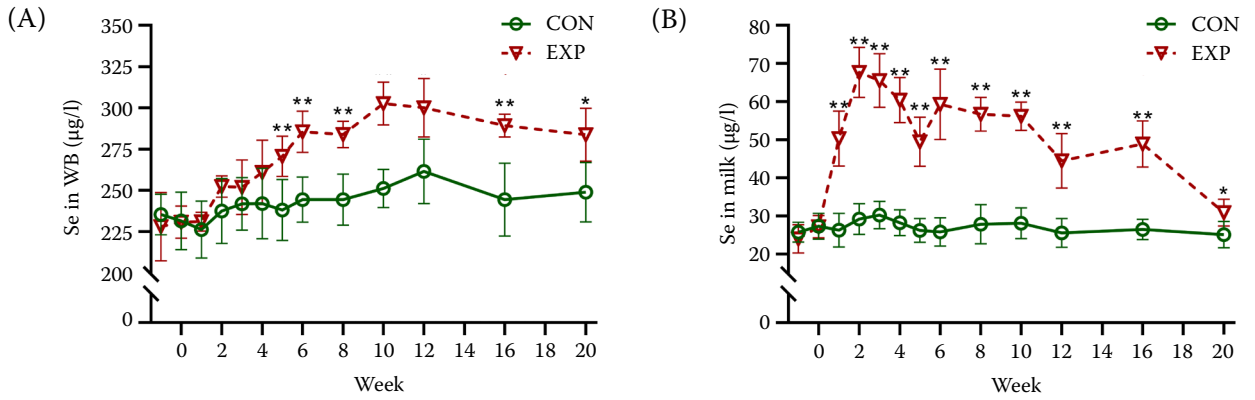

Figure 1. Selenium concentration in (A) whole blood and (B) milk samples from cows during a 22-week supplementation trial

Asterisks indicate statistically significant differences between the CON and EXP groups for a given sampling week: *$P < 0.05$, **$P < 0.005$

CON = control group; EXP = experimental group; WB = whole blood

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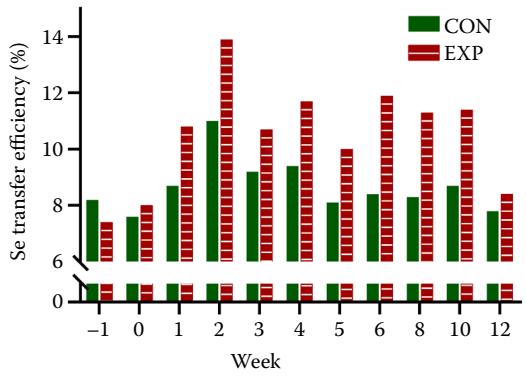

Figure 2. Transfer efficiency of dietary Se to milk calculated as the ratio of Se intake to Se yield in milk for control and experimental cows during the first 12 weeks
CON = control group; EXP = experimental group

The efficiency of Se transfer from diet to milk in EXP animals was 11.1 ± 1.5% during the first 12 weeks of supplementation, compared to 8.8 ± 1.0% in CON animals (Figure 2). The highest efficiency was observed in cows receiving Se-enriched silage, reaching 13.9% in wk(2). This parameter stabilised at 11.1 ± 0.7% from wk(3) to wk(10), followed by a pronounced decline to 8.4% by wk(12).

Antioxidant status of dairy cows

The temporal changes in parameters characterising the antioxidant status of the animals, as determined by *in vitro* blood analysis, are presented in Figure 3A–F. The statistical analysis did not re-

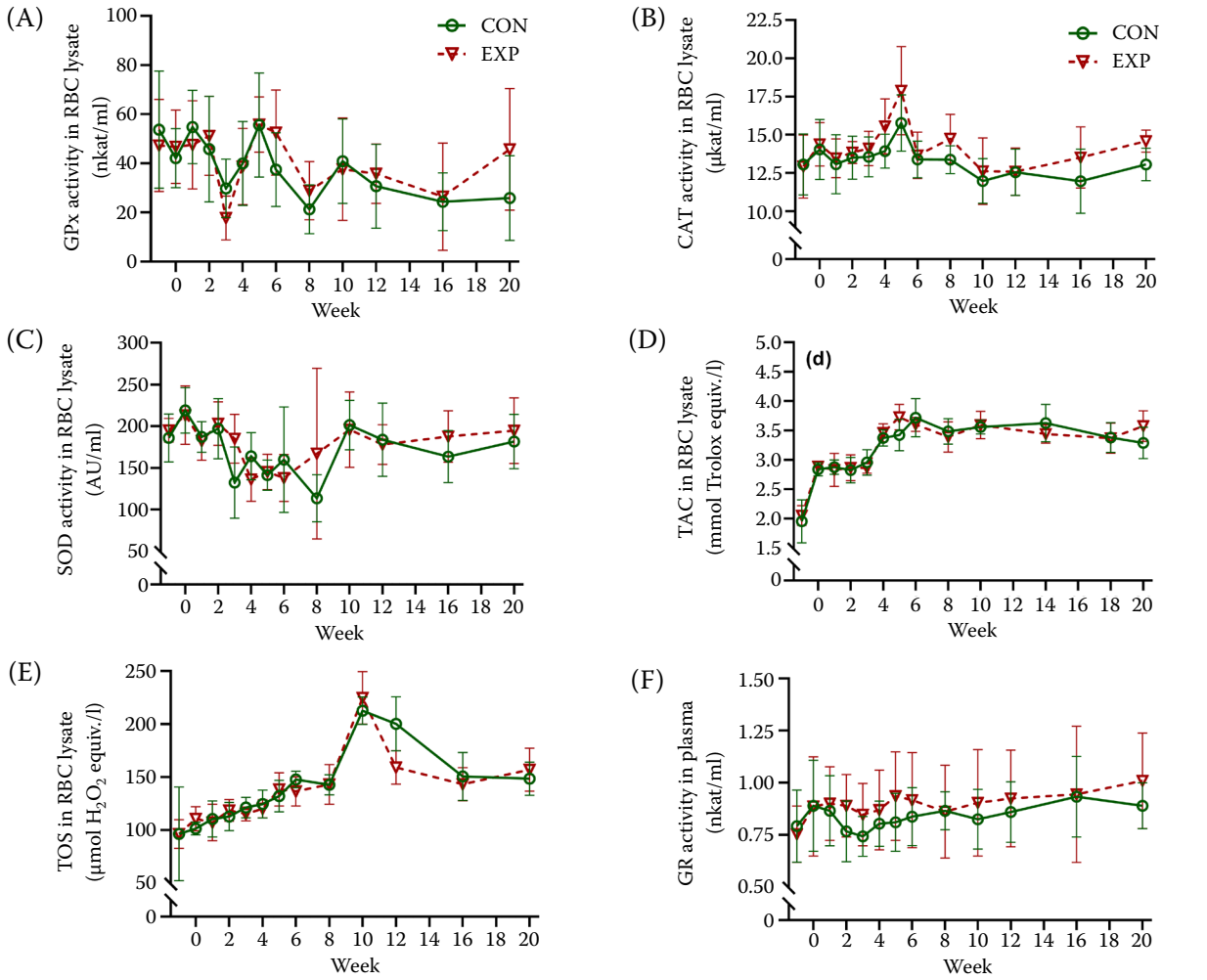

Figure 3. Antioxidant status of cows as determined in red blood cell (RBC) lysates and plasma
(A) GPx activity; (B) CAT activity; (C) SOD activity; (D) total antioxidant capacity (TAC); (E) total oxidation status (TOS) in RBC lysates; and (F) GR activity in plasma during a 22-week supplementation trial
CAT = catalase; CON = control group; EXP = experimental group; GPx = glutathione peroxidase; GR = glutathione reductase; SOD = superoxide dismutase

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veal any significant differences in GPx, CAT, SOD, TAC, TOS, and GR between the CON and EXP groups during the observed weeks. High variability in enzyme activities among individual cows within both the CON and EXP groups impeded the precise evaluation of general trends. In the CON group, there was a decreasing trend in GPx activity over time, beginning at 53.7 ± 23.9 nkat/ml in wk(–1) and ending at 25.9 ± 17.2 nkat/ml in wk(20), with two local minima observed in wk(3) and wk(8). The SOD activity in CON cows experienced a decline between wk(3) and wk(8), with local minima of 132 ± 43 and

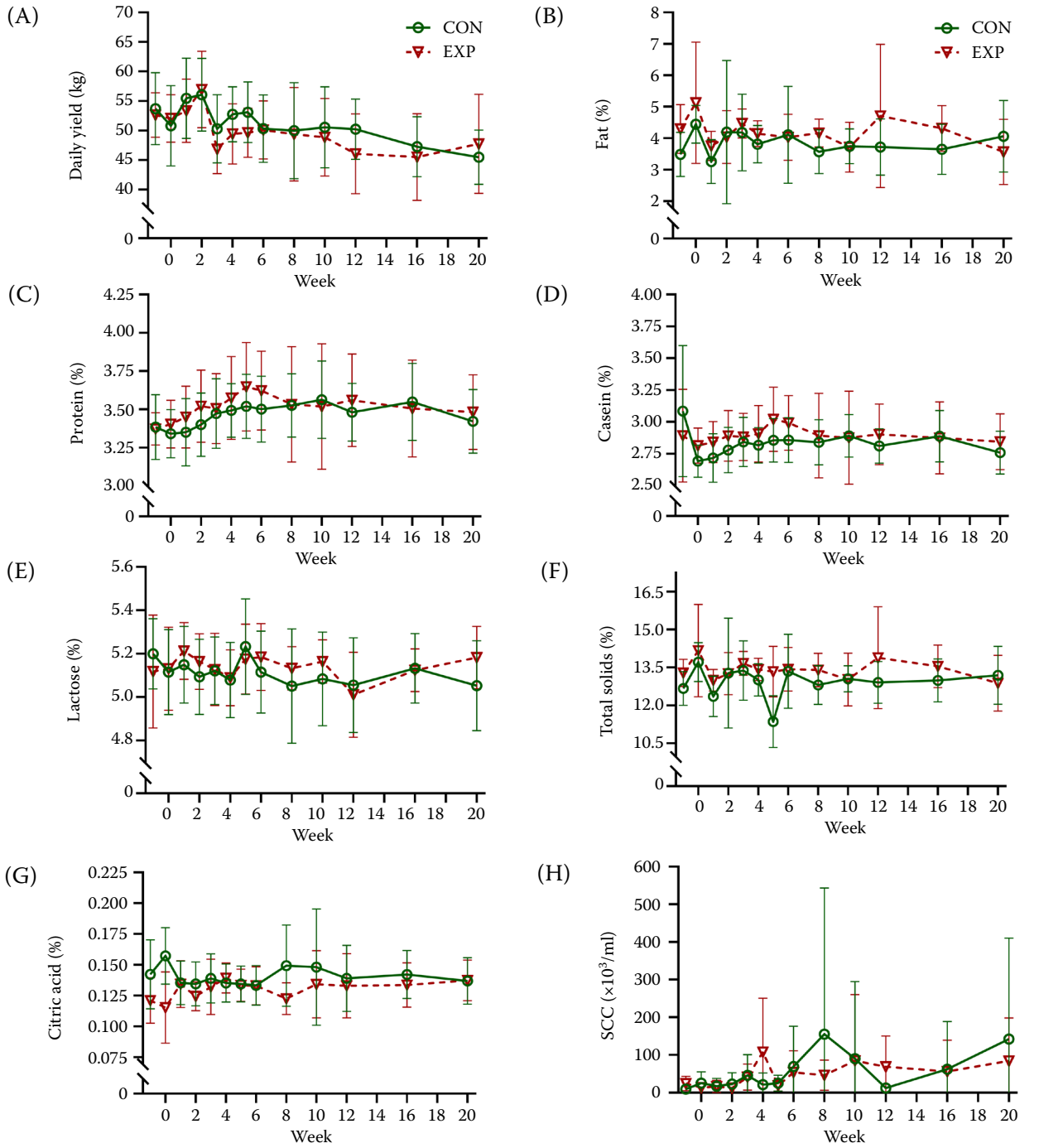

Figure 4. Milk performance of control (CON; green circles) and experimental (EXP; red triangles) cows over a 22-week supplementation trial

(A) Daily milk yield and milk composition: (B) fat, (C) protein, (D) casein, (E) lactose, (F) dry matter, (G) citric acid, and (H) somatic cell count in milk (SCC)

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113 ± 28 AU/ml, before rising to 201 ± 30 AU/ml by wk(10), which is consistent with pre-decline levels. For the CON group, CAT and GR activities remained stable over time, with the average values of 13.3 ± 1.8 μkat/ml and 0.84 ± 0.15 nkat/ml, respectively. Minor deviations from this trend included a local maximum in wk(5) for CAT and a local minimum around wk(3) for GR. TAC levels sharply increased from 1.95 ± 0.37 mmol Trolox equiv./l in wk(–1), reaching a plateau at 2.87 ± 0.17 mmol Trolox equiv./l between wk(0) and wk(3), and then plateauing again at 3.48 ± 0.27 mmol Trolox equiv./l from wk(4) to wk(20). TOS levels gradually increased from 96.3 ± 44.3 μmol H_2O_2 equiv./l in wk(–1), sharply peaking at 213 ± 13 μmol H_2O_2 equiv./l in wk(10), followed by a continuous decline. Overall, the EXP group exhibited similar patterns to the CON group, with a tendency to maintain higher levels of CAT and GR activities.

Milk yield and composition under Se supplementation

The effects of Se_{tot} and dietary Se sources in TMR on daily milk yield and milk composition over time are illustrated in Figure 4. No significant differences were observed between the CON and EXP cows in milk yield, fat content, protein, casein, lactose, total solids, urea, citric acid, and SCC during any sampling week. Although some fluctuations in these parameters were noted over time in both treatments, the mean values were within the normal range and were not further investigated in this study. The most critical aggregated data for the supplementation period were as follows: fat (3.83 ± 1.13% vs 4.09 ± 0.97%), protein (3.48 ± 0.21% vs 3.54 ± 0.28%), lactose (5.11 ± 0.19% vs 5.14 ± 0.14%), and daily milk yields (51.0 ± 6.3 kg vs 49.5 ± 6.7 kg) for CON and EXP cows, respectively. Note that data from the pre-supplementation period, wk(–1) and wk(0), were excluded.

DISCUSSION

Interpreting the common Se status indicators, such as total Se concentration, GPx activity, and other Se-dependent proteins across various matrices, including whole blood, blood components, and milk, requires insight into the hierarchy of selenoproteins and the distinct role of dietary SeMet. SeMet can be integrated into the methionine pool of the body and nonspecifically incorporated into polypeptide chains (Burk and Hill 2015). As a result, SeMet-rich feed, such as biofortified silage, is expected to induce a different response in these indicators compared to Se^{IV}.

Selenium-dependent indicators in blood

Using Se concentration and GPx activity in WB as indicators of the Se status in cattle has some limitations, particularly due to their low sensitivity to short-term changes in dietary Se intake, often exhibiting delayed responses (Gerloff 1992). A meta-analysis of 29 studies by Respati et al. (2023) highlights a consistent trend of increasing Se concentrations in WB with higher Se intake in dairy cows, with Se_{org} forms showing greater efficiency than inorganic forms. However, no similar dose-response relationship was observed for GPx activity in lactating cows, even when Se_{org} was administered, which aligns with our findings (Figure 3A). In our study, the erythrocyte GPx activity did not differ significantly between CON and EXP cows on any of the sampling days. These results are consistent with those of Calamari et al. (2010), who also reported no significant effect of Se source or dose on this indicator in WB or plasma. A significant effect of the Se form administered to cattle was more frequently observed for WB selenium concentration than for GPx activity, as summarised by Weiss (2003). The author attributed the higher efficiencies of selenium accumulation in WB to differences in Se metabolism pathways between selenite- and SeMet-based diets. Similarly, Seboussi et al. (2016) found a significant effect of Se dose (0.1–0.9 mg/kg Se in DM) and Se form (selenite vs SY and Se-biofortified silage) on WB selenium levels, but no effect on GPx activity. In our trial, the Se concentration in WB of the CON group consistently exceeded 220 μg/l, surpassing the 100 μg/l minimum required for optimal immune function and fertility, and even meeting the 200 μg/l threshold for resistance to infectious mastitis, as explained by Calamari et al. (2010). These Se levels reflect the intensive Se supplementation strategy (0.6 mg/kg Se in DM of TMR) employed on the high-yield dairy farm under study. Administration of biofortified silage (0.9 mg/kg Se in DM of TMR), which provided roughly 50% more Se_{tot} to EXP animals, re-

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sulted in a 17% increase in WB selenium in wk(6–12), a statistically significant change ($P < 0.05$). Literature suggests that both Se dose-response and Se form-response effects are significant only when the supplementation transitions cattle from Se deficiency to adequacy. Knowles et al. (1999) observed an 880% increase in the whole blood Se concentration after 133 days of supplementation (2 or 4 mg Se per cow per day as Se^{VI} or SY) compared to control animals with 17 µg/l Se in WB (0.55 mg Se per cow per day from pasture). Gong et al. (2014) found no notable effect of Se form (Se^{IV} vs SY at 0.3 mg/kg Se in TMR) on whole blood Se concentration (121–135 µg/l) or serum GPx activity. Azorin et al. (2020) supplemented TMR with 0.24 mg/kg Se, reporting WB Se concentrations of 130 µg/l (Se^{IV}) and 159 µg/l (60/40 inorganic/organic Se mix) by day 49, with negligible differences in GPx activity between treatments. Moreover, Ivancic Jr and Weiss (2001) found no effect of Se dose in TMR (0.1 mg/kg to 0.3 mg/kg Se as Se^{VI}) on the WB GPx activity by day 112. Phipps et al. (2008) observed a peak WB Se level (283 µg/l), comparable to that in our study (262 ± 20 µg/l for CON animals), with all measurements taken in wk(12). However, Phipps' group used 0.45 mg/kg Se in SY-based TMR, whereas our CON cows received primarily selenite. There exist discrepancies in the literature regarding the Se concentration plateau in WB during supplementation, with Se dose appearing to be the primary determinant. In our study, the plateau was reached in wk(10) for EXP cows, consistently with Ortman and Pehrson (1999), who showed a plateau with Se^{IV} or Se^{VI} supplementation (0.24–0.31 mg/kg Se in TMR), though Se levels continued to rise beyond wk(10) with SY. Calamari et al. (2010) demonstrated that 11 weeks were required to reach 95% of the asymptotic WB selenium concentration, while Juniper et al. (2008) observed an apparent plateau within six weeks with a very high SY dose (6.25 mg/kg Se in TMR). However, whole blood Se does not always stabilise within experimental periods; Knowles et al. (1999) calculated that 95% of the asymptotic value would take nearly 40 weeks to reach, though this estimate may carry significant uncertainty.

Selenium in milk

A meta-analysis by Ceballos et al. (2009), encompassing 42 studies from 1977 to 2007, identified Se source and dose as the primary factors influencing Se concentration in milk during supplementation studies, with minor effects from lactation stage and geographical factors. In our study, CON cows exhibited stable Se levels in milk (27 ± 4 µg/l) when fed Se close to the permissible limit. Consistently with our findings, Se concentrations ranging from 20 µg/l to 40 µg/l are typical on the farms that closely adhere to the European legal limit when inorganic Se salts are used (Phipps et al. 2008; Gong et al. 2014). Several studies have demonstrated that SY effectively increases Se levels in milk within the common supplementation range of 0.2–0.5 mg/kg Se in TMR (Phipps et al. 2008), though there exist exceptions, such as Barbe et al. (2020), where cows showed negligible differences in milk Se concentrations between herds fed 0.2 and 0.3 mg/kg Se (SY) on a basal diet containing 0.3 mg/kg Se^{IV}. Crucially, inorganic Se^{IV} and Se^{VI} stagnate in contributing to milk Se enrichment (Knowles et al. 1999; Ortman and Pehrson 1999; Azorin et al. 2020). The Se transfer to milk was highly efficient, as a 53% increase in Se_{tot} in the TMR resulted in a 132% increase in milk Se concentrations, reaching 68 µg/l in wk(2). Some studies have explored the cows' capacity for milk fortification, with concentrations of 166–247 µg/kg Se reliably achieved on several farms (Doyle et al. 2011). Sun et al. (2021) recorded levels as high as 583 µg/kg Se after supplementing 5 mg/kg Se in the form of SY in TMR. This high scalability in response to SY may be attributed to the substitution of SeMet for methionine, which is abundant in milk proteins, when feeding SY rich in SeMet (Weiss 2003). In concordance, Sun et al. (2021) identified a high proportion of SeMet (91% of Se_{tot} content) in fortified milk using HPLC-ICP-MS. Using the same analytical technique, Calamari et al. (2010) found that SeMet concentrations increased with SY supplementation, but not with Se^{IV}. Notably, Seboussi et al. (2016) administered 0.84 mg/kg Se (predominantly as selenite) in TMR and 0.89 mg/kg Se in Se-enriched silage, resulting in milk Se concentrations of 28 µg/l and 60 µg/l, respectively, which were strikingly similar to those measured in our trial (27 µg/l and 68 µg/l for CON and EXP cows, respectively).

The time required to reach a plateau in milk Se concentration is a critical technological parameter for producing Se-enriched dairy products. In our trial, the EXP group reached a maximum concentration of 68 ± 7 µg/l Se in wk(2). However, Se con-

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centration exhibited a continuous decline, dropping to 49 ± 6 µg/l by wk(16). Rapid plateauing of milk Se concentrations within the first or second week was reported by Doyle et al. (2011) on multiple farms. Some authors did not observe an increasing trend in milk Se content beyond the second week (Barbe et al. 2020) and the third week (Azorin et al. 2020) of supplementation; however, their evaluations were concluded after five weeks and seven weeks, respectively. In contrast, Phipps et al. (2008) did not observe a peak in milk Se levels by day 84 in certain treatments, and Gong et al. (2014) reported a significant difference in Se levels between days 30 and 60. To properly investigate the stability of Se levels in milk after reaching the peak, longer time series are evidently required. In contrast to our findings (Figure 1B), the literature shows Se levels in milk remaining near the plateau concentrations (Calamari et al. 2010). We hypothesise that the decline in Se levels observed in our trial may be partly due to the extended storage time of the silage as it was progressively unloaded from the bag. Inorganic Se is known to undergo transformation in silage, potentially forming nano-sized elemental selenium (nSe^0), which is presumed to have reduced bioavailability (Lee et al. 2019). Unfortunately, data on Se transformation during the ensiling process and its impact on bioavailability are still limited. Our results support the formation of nSe^0 only indirectly, as decreasing recovery of Se was noted in the speciation analysis of silage samples taken over time (data not shown).

While Se concentration in milk is a crucial parameter for producers of Se-enriched dairy products, the efficiency of Se transfer from diet to milk is an important indicator of the economic cost of fortification. In the present study, the transfer efficiency of dietary Se to milk in cows supplemented with Se^{IV} in the basal (CON) diet was 8.8 ± 1.0%, which is notably higher than the 3.2–4.6% reported for Se^{IV} in other studies (Calamari et al. 2010; Seboussi et al. 2016). However, caution is needed when interpreting Se transfer metrics, as different studies apply varying definitions. For example, Calamari et al. (2010) used adjusted efficiencies corrected for Se present in the basal diet. When recalculated based on Se_{tot} intake per cow and day, the efficiencies were found to range from 7% to 10%. Ivancic Jr and Weiss (2001) achieved Se transfer efficiencies of 6.1–8.1% for Se^{VI} administered at 4–5 mg per cow per day, increasing to 16–19% at lower supplementation levels of 2–3 mg Se, highlighting the substantial impact of marginal Se doses. For Se supplementation with SY at adequate or supranutritional levels, the literature reports a broad range of efficiencies, from 8% to 20% (Calamari et al. 2010; Doyle et al. 2011; Seboussi et al. 2016). Notably, Barbe et al. (2020) achieved a high efficiency (20%) when administering TMR containing a combined Se source of 0.3 mg/kg Se from Se^{IV} and 0.3 mg/kg from SY. In contrast, our trial employed a mixed diet with the total Se content of 0.9 mg/kg (0.6 mg/kg primarily from Se^{IV} and 0.3 mg/kg from Se-enriched silage), resulting in a maximum transfer efficiency of only 13.9%. These findings emphasise that both the form and the dosage of Se are critical factors influencing Se transfer to milk. However, other variables, such as diet composition (Ivancic Jr and Weiss 2001), daily milk yield (Calamari et al. 2010), and herd management practices (Doyle et al. 2011), also play significant roles and are beyond the scope of this study.

Milk yield, composition, and antioxidant response to Se supplementation

Our findings align with the general consensus that Se dosage and source are unlikely to consistently and reliably enhance milk yield, milk composition (fat, protein, and lactose content), or reduce SCC (Gong et al. 2014; Azorin et al. 2020; Barbe et al. 2020).

In our study, no significant differences were observed between the CON and EXP groups in milk yield, composition, or SCC across any sampling week. Although some fluctuations in these parameters occurred during the trial (Figure 4), we attribute them to inherent physiological variations in high-yielding dairy cows and a degree of data variability. However, positive effects of Se supplementation on specific individual parameters have been frequently documented in the literature. Sun et al. (2021) noted a trend toward higher milk yields, and statistically significant effects of Se supplementation have also been reported (Phipps et al. 2008). Higher dietary Se levels or the inclusion of SY significantly reduced SCC and mastitis incidence (Sun et al. 2021) and increased milk fat content (Knowles et al. 1999). Respati et al. (2023) suggested that discrepancies in trial duration, diet

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composition, previous Se status, lactation stage, and management or stress factors may account for the inconsistent results reported regarding selenium effects on milk performance.

While enzymatic antioxidants such as GPx, SOD, and CAT represent the primary form of intracellular antioxidant defence, TAC serves as a comprehensive index to reflect the ability to counteract external stress and manage free radicals (Gong and Xiao 2018). In our study, no significant differences were observed between the CON and EXP groups in CAT, SOD, GR, or TAC (Figure 3A–F) activities. This may be attributed to the absence of notable stress level differences between the groups, as indicated by TOS (Figure 3E). Moreover, the basal diet provided sufficient Se intake, rendering additional Se supplementation ineffective in eliciting significant changes in the monitored indicators. A dairy trial by Gong et al. (2014) reported increased CAT, SOD, thioredoxin reductase, and TAC, while Azorin et al. (2020) observed a reduction in TOS. Both trials involved replacing Se^{IV} with SY, at least partially, in TMR, but importantly, the Se supplementation levels were relatively low at 0.3 and 0.24 mg/kg, respectively. Similarly, Li et al. (2019) documented elevated SOD activity and TAC in the plasma of cows as Se doses increased from 0.04 mg/kg to 0.5 mg/kg in diets supplemented with Se_{org}. However, consistently with our findings, Sun et al. (2021) did not observe any effects on SOD or TAC in the serum of cows receiving Se supplementation at 0.5 and 5 mg/kg in TMR as part of a supranutritional trial.

CONCLUSION

For dairy farms seeking the commercial production of Se-enriched milk and dairy products, direct Se analysis in milk is an indispensable indicator for monitoring supplementation, particularly regarding the initiation, peak levels, and long-term stability of Se concentration. The substitution of conventional low-Se maize silage with Se-enriched silage in a total mixed ration already supplemented with selenite (0.6 mg/kg Se in DM), thereby raising the Se_{tot} to 0.9 mg/kg Se in DM, proved to be an effective strategy for significantly increasing Se levels in milk beyond what is achieved through selenite supplementation alone. Under the dietary Se conditions applied, no adverse effects were observed on milk composition, daily yield, somatic cell counts, or antioxidant status, as indicated by blood-based markers including GPx, SOD, CAT, GR, TAC, and TOS. A rapid peak in milk Se concentration (68 µg/l) was reached within two weeks of supplementation, with the Se transfer efficiency to milk of up to 13.9%, compared to 8.8% observed with selenite supplementation alone. However, the gradual decline in both the milk Se concentration and the transfer efficiency after attaining the peak values warrants further investigation. Future studies should focus on the transformation of Se, particularly on the formation of nano- or colloidal-sized elemental Se^0 during the ensiling process, as this is suspected to decrease Se availability in the diet. Additionally, the feasibility of fully replacing selenite with high-Se silage as the exclusive dietary Se source requires thorough evaluation.

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Conflict of interest

The authors declare no conflict of interest.

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