

RESEARCH ARTICLE

Effects of trisodium citrate and reduced levels of nanominerals supplementation on subclinical mastitis affected dairy cows

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Abstract: Trisodium citrate (TSC) and trace minerals like Zinc (Zn), copper (Cu), and manganese (Mn) play an important role in maintenance of bovine udder health and immunity. Present study evaluated the effect of co-administration of TSC and reduced levels of nanominerals (Zn, Cu, Mn) on the recovery of Subclinical Mastitis (SCM) affected Holstein Friesian (HF) crossbred cows. Nanominerals were synthesized by chemical reduction methods and characterized by Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray and Spectroscopy (EDS) methods. Cows diagnosed as SCM, based on milk Somatic Cell Count (SCC; $e^{200} \times 10^3$ cells/ml; $n=8$), were additionally co-administered (orally) with TSC (30 mg/kg/day) and half of the recommended levels of nanominerals (Cu: 6.5 ppm, Mn: 8.75 ppm and Zn: 30 ppm/kg DMI/day) for 28 days and the effects were compared with SCM affected cows ($n=6$) and apparently healthy cows (SCC $d^{200} \times 10^3$ cells/ml; $n=8$) provided with only basal diet. Data were analyzed by mixed-model procedure and McNemar test. The particle sizes of the nanominerals were < 100 nm. Co-administration of TSC and nanominerals had no significant effects on milk yield, its composition and superoxide dismutase (SOD) levels but, marginally reduced milk SCC and Californian Mastitis Test (CMT) score. Similarly, co-supplemented cows had higher cure rate against *Streptococcus spp.*-mediated infections, but not reduced the *Staphylococcus spp.*- or Coliform-mediated intra mammary infections. It is concluded that co-administration of TSC with half of the recommended levels of nanominerals had no

biologically convincing effects on recovery of SCM affected HF crossbred cows.

Keywords: Nanominerals; Trisodium citrate; Scanning Electron Microscopy; Somatic cell count; Bacteriological cure rate; Subclinical mastitis

Introduction

Mastitis is the most common disease in dairy cattle and causes more economic losses to dairy farmers through reduction of milk yield, milk quality, treatment and culling cost. The incidence of subclinical mastitis (SCM) is more common (40-63%) than clinical mastitis (CM: 6-38%) among Indian dairy animals (Krishnamoorthy et al. 2021). Two decades back, an annual economic loss to Indian dairy industry due to SCM and CM mastitis was about 41500 to 43650 million and 17000 to 30000 million, respectively (Wani et al. 2022). The economic loss per lactation is more in high yielding crossbred cows than indigenous dairy cattle (Rathod et al. 2017). Mastitis is also the most important reason for antimicrobials usage in dairy animals, which is an important public health concern for development of antimicrobial resistant pathogens (Sharma et al. 2022). In order to reduce the antibiotic usage, it is important to reduce the susceptibility to mastitis through optimization of housing, nutrition and immunity of dairy animals. Indeed, nutritional management through supplementation of antioxidant and immunity specific trace

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minerals are common practices to improve udder health of dairy animals (McMullen et al. 2021).

Copper (Cu), Zinc (Zn) and Manganese (Mn) are most critical trace minerals and play an important role in maintenance of udder health and immunity of dairy animals (Sahu et al. 2023). The immune system is affected first during mineral deficiency before showing impact on growth and fertility (Gombart et al. 2022). On the other hand, supplementation of trace minerals during peripartum period resulted in improved functionality of neutrophils and reduced severity of mastitis (Libera et al. 2021). However, the micronutrients contents vary in feedstuff and water, and their absorption and utilization are affected by their source and patho-physiological status of animals (Byrne and Murphy 2022; Cha et al. 2025). Thus, the current global recommendation of trace minerals may not be sufficient (Socha et al. 2006). In this situation, supplementation with higher level or more bioavailable mineral sources are the possible alternatives. Of which, the former strategy may not be suitable due to higher cost, lesser safety margin for some minerals (e.g., Cu), and environmental contamination. On the other hand, nanominerals can effectively fulfill the requirement at reduced dosage due to their more bioavailability (Swain et al. 2023). They indicated that 50% of the recommended level of nano Zn had similar or better effects than inorganic Zn at their 100% of recommended levels in terms of bioavailability, immunity, metabolic hormones levels and superoxide dismutase (SOD) gene expression level in rats and goats.

Citrate is a major precursor for milk synthesis, which helps to maintain normal pH of milk at 6.5 and keeps milk fluidity through sequestration of free Ca^{2+} in milk (Shennan and Peaker 2000). As a result, a drop in milk's citrate level causes Ca^{2+} aggregation, which in turn damages mammary tissue through inflammatory reactions (Dhillon and Singh 2013). Dhillon et al. (1995) observed beneficial effects of trisodium citrate (TSC) supplementation in mastitis affected animals. Additionally, Sahu et al. (2022) also reported that supplementation of TSC alone can lower the California Mastitis Test (CMT) score and milk Somatic Cell Count (SCC) in SCM affected cows. Supplementation of TSC in combination with minerals is a common practice for SCM affected animals in India (National Dairy Development Board Annual Report 2018). On the other hand, studies indicated that several factors influence the milk citrate level and blood-milk barrier prevents the exchange of citrate in both directions (Akkerman et al. 2019; Dunshea et al. 2019). It warrants further studies to understand the effects of TSC supplementation in SCM affected animals. We hypothesized that replenishment of milk citrate levels and improving blood mineral levels through nanominerals supplementation could improve the udder health in SCM affected cows. Accordingly, the present study aimed to evaluate the effect of co-administration of TSC and nanominerals (Cu, Zn and Mn) at 50% of the recommended levels on recovery in SCM affected dairy cows.

Materials and methods

Synthesis and characterization of nanominerals

Nano zinc oxide (ZnO), nano copper oxide (Cu_2O), and nano manganese oxide (MnO_2), were synthesized by chemical reduction methods and characterized by Scanning Electron Microscopy (SEM), Energy Dispersive X-Ray Spectroscopy (EDS), Transmission Electron Microscopy (TEM) and X-Ray Diffraction (XRD) techniques.

Study animals

The experiment was conducted at the Livestock Research Centre, Southern Regional Station of ICAR- National Dairy Research Institute in Bengaluru, Karnataka, India. Lactating HF crossbred cow's (n=22) milk samples were screened for SCM using SCC and CMT for three days and cows diagnosed as healthy or SCM based on milk SCC were grouped into three based on milk yield, parity, and lactation stage. SCM-affected cows ($\text{SCC} \geq 200 \times 10^3 \text{ cells/mL}$) in group 1 (n=8) were orally administered with TSC (30 mg/kg b.wt/day) and half of the recommended levels of nanominerals (Cu: 6.5 ppm, Mn: 8.75 ppm, Zn: 30 ppm/kg DMI/day) for 28 days before morning milking on an empty stomach. Group 2 (n=8) consisted of healthy cows ($\text{SCC} \leq 200 \times 10^3 \text{ cells/mL}$), while group 3 (n=6) comprised SCM-affected cows without additional supplementation. The experiment was conducted as per approval and guidelines of Institutional Animals Ethics Committee (IAEC).

Feeding and housing management of study animals

Study animals were fed as per NRC (2001) recommendations with a mixture of green fodders, ragi straw and commercially available concentrate mixture (Nandini gold™ cow feed, M/s Karnataka Milk Federation, Bengaluru). Concentrate was given twice in a day during morning and evening milking periods. Study animals were sheltered in the Mangalore tiles based roof and concrete-enforced stone flooring system with appropriate space as per Bureau of Indian Standard guidelines.

Milking management and milk sampling

Study animals were milked twice in a day using bucket type machine milking system and daily milk yield (in kg) of each cow was estimated by electronic weighing balance during 7 days prior to study period, 28 days of experiment period and 14 days after study period.

About two to three initials strips of milk were discarded from each quarter and CMT was performed as per manufacturer's instruction (M/s Immucell Corporation, USA). About 6-8 mL of pooled milk samples of each cow were collected during morning milking period for estimation of milk composition (fat, SNF, protein, lactose and ash) and milk SCC using ultrasonic milk

analyzer (M/s Vector, Karnataka, India) and DeLaval Cell Counter (M/s DeLaval, Sweden), respectively.

Milk culturing

About 8-10 mL of pooled milk samples from each cows were collected aseptically following National Mastitis Council guidelines (NMC 2004) and processed for culturing within 2 hours. Briefly, one mL of milk sample with 9 mL of nutrient broth was incubated at 37°C for 24 hours. Milk samples shown growth were further inoculated in selective media such as MacConkey agar (*Coliform*), Edwards agar (*Streptococcus spp.*), and Mannitol salt agar (*Staphylococcus spp.*) at 37°C for 24 hours. Bacterial isolates were identified based on gram staining and morphological characteristics. All the chemicals were procured from M/s Himedia, Mumbai (India).

Estimation of milk citrate level

Milk citrate level was estimated as per Ali et al. (1998) with little modification. Briefly, the reaction mixture containing 10 ml of copper chloride solution (pH 6.5), one ml of milk and 9 mL of distilled water was incubated at 40°C for 5 minutes to form blue coloured complex. The absorbance was taken at 760 nm using UV spectrophotometer (M/s Hitachi, Japan).

Blood sampling for estimation of plasma minerals and SOD levels

About 4 mL of blood samples were collected from jugular vein using EDTA containing vacutainer tubes, centrifuged at 1500 g for 20 min at 4°C, plasma was separated, and stored at -20°C till estimation of Zn, Cu, Mn by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Optima 8000, M/s Perkin-Elmer, USA) after mild acid digestion (Seo et al. 2014). Plasma and milk SOD levels were estimated using bovine specific ELISA kit (M/s Genetix Biotech Asia Pvt. Ltd., New Delhi, India).

Statistical analysis

Data were analyzed using a mixed-model procedure in which group, time, and their interaction were considered as fixed factors. Univariate method used for analysing the interactions between groups or within a group at each time interval. The difference of intra-mammary infection (IMI) rates between groups were analysed by McNemar test. Statistical analysis was done using SPSS Statistics version 20.0 software.

Results and Discussion

Characterization of nanominerals

TEM analysis indicated sizes of approximately 30 to 40 nm for ZnO (Supplementry Fig. S1a), 51 to 84 nm for Cu₂O (Fig. S1b), and 8 to 16 nm for MnO₂ (Fig. S1c). SEM images showed spherical

shapes for Zn and Cu nanominerals and rod shapes for Mn, with sizes ranging from 39 to 90 nm (Fig. S2a), 23 to 88 nm (Fig. S2b), and 24 to 52 nm (Fig. S2c), respectively. The EDX spectrum confirms the presence of Zn (Fig. S3a), Cu (Fig. S3b) and Mn (Fig. S3c) in ZnO, Cu₂O and MnO₂ respectively. The mean crystalline size of ZnO, Cu₂O and MnO₂ nanominerals determined by XRD method and subsequent calculation using the Debye Scherrer formula were 22 nm (Fig. S4a), 11 nm (Fig. S4b) and 49 nm (Fig. S4c) respectively. XRD patterns of ZnO, Cu₂O, and MnO₂ nanominerals showed peak broadening, confirming their small size. Comparison with standard JCPDS cards for metallic nanoparticles of ZnO, Cu₂O, and MnO₂ revealed strong correlation, validating the identification of these nanominerals.

Effect of co-administration of TSC and nanominerals on average weekly milk yield and its components in SCM affected cows

Co-administration of TSC and nanominerals in SCM-affected cows had no significant effect on milk yield (Fig. 1) or its composition (Fig. S5) compared to healthy cows. Recently, we reported that supplementation of TSC alone in SCM affected cows cause no significant changes in milk yield and its composition (Sahu et al. 2022). Supplementation of bicarbonate, a citrate metabolite also did not alter milk yield and fat content in lactating cows (Wittayakun et al. 2015; Stumpf et al. 2013). Dietary supplementation of nano Zn during peri-partum period cause no significant change in milk yield and its components in goats (Shafi et al. 2020). A meta-analysis study also indicated no influence of Zn supplementation on milk components in dairy cows (Kusuma et al. 2025)

Effect of co-administration of TSC and nanominerals on milk SCC and CMT score in SCM affected cows

Co-administration of TSC and nanominerals resulted in marginal reduction ($G \times T$; $P=0.001$) of milk SCC (5%) and CMT score (32%) in SCM-affected cows compared to non-supplemented SCM-affected cows (Table 1). Non-supplemented SCM-affected cows showed increase in these udder inflammatory indicators after the supplementation period, indicating persistent or new IMI. Biologically non-significant reduction of milk SCC in treated group indicates supplementation had little impact on SCM. Simialry, Kumar et al. (2009) reported that inorganic form of Cu or Zn supplementation before calving had no or little impact on recovery from SCM after calving. Ganda et al. (2016) also reported that administration of trace minerals in cows with elevated SCC had no effect on milk yield, milk components and milk SCC as also observed in this study. In contrast, studies also indicated that significant reduction of milk SCC after supplementation of different sources of Cu and Zn in goats and dairy cattle (Shafi et al. 2020; Oconitrillo et al. 2024).

Effect of co-administration of TSC and nanominerals on intra-mammary infection rate

Co-administration of TSC and nanominerals significantly reduced the intra-mammary infection rate (IMIR) mediated by *Streptococcus spp.* by 50% ($P \leq 0.05$) in SCM-affected cows (Table 2). However, there was no significant reduction of IMIR by *Staphylococcus spp.* and *Coliform*. In Karnataka (India), *Staphylococcus spp.* are more commonly associated with SCM affected cows (Kurjogi and Kaliwal 2011). This indicated that co-administration of TSC and nanominerals had no consistent or biologically significant impact against major mastitis pathogens in SCM-affected cows. The observed trends of milk SCC and CMT score in these animals further supports the bacterial culture findings. Although, few studies reported the reduced bacterial colony count in TSC-supplemented buffaloes, the effect on bacteriological cure remains unclear (Dhillon et al. 1995; Yousaf et al. 2010). Several studies indicated that mineral supplementation

including Zn as organic or nano forms caused no impact on IMIR (Hosseini-Vardanjani et al. 2020).

Effect of co-administration of TSC and nanominerals on milk citrate level in SCM affected cows

Co-supplementation of TSC and nanominerals had no significant effects on milk citrate level (Fig. S6) as observed earlier in TSC alone supplemented cows (Sahu et al. 2022). However, other studies reported increased milk citric acid levels after TSC supplementation in mastitis-affected dairy cattle (Dhillon et al. 1995; Singh et al. 1997). Milk citrate levels vary with lactation stage, milk yield, constituents, season, and diets (Akkerman et al. 2019; Dunshea et al. 2019). Minimal variation in lactation stage along with no significant changes in milk yield or composition in this study could be the possible reason for lack of significant effects on milk citrate levels.

Table 1: Effect of co-administration of trisodium citrate (TSC) and nanominerals (Nano) on udder health indicators and plasma minerals level in crossbred cows

	Milk SCC (In log)		CMT Score		Plasma Zn level (mg/L)		Plasma Cu level (mg/L)		Plasma Mn level (mg/L)	
	Before	After	Before	After	Before	After	Before	After	Before	After
TSC+ nano	9.70 ± 0.27	9.65 ± 0.32	2.38 ± 0.31	1.63 ± 0.31	0.52 ± 0.09	0.25 ± 0.03	0.68 ± 0.11	0.28 ± 0.03	0.05 ± 0.01	0.09 ± 0.01
Healthy	6.94 ± 0.27	7.37 ± 0.32	0.00±0.00	0.00±0.00	0.45 ± 0.09	0.18 ± 0.03	0.59 ± 0.12	0.10 ± 0.03	0.06 ± 0.01	0.08 ± 0.01
SCM	8.57 ± 0.32	9.08 ± 0.37	0.83 ± 0.36	3.00 ± 0.36	0.22 ± 0.10	0.07 ± 0.04	0.16 ± 0.12	0.06 ± 0.03	0.09 ± 0.01	0.09 ± 0.01
P Value	G: 0.31; Time: 0.001; GxT: 0.54		G: 0.001; Time: 0.26; GxT: 0.001		G: 0.31; Time: 0.001; GxT: 0.54		G: 0.15; Time: 0.001; GxT: 0.53		G: 0.02; Time: 0.001; GxT: 0.01	

TSC+Nano: SCM affected cows co-administrated with TSC and nanominerals (n=8), Healthy cows (n=8), SCM: Subclinical Mastitis (n=6). Value are expressed as least square means±SE in different groups during before (day 0) and after (day 28) treatment period. Fixed factors having P<0.05 is statistically significant.

Table 2: Effect of co-administration of trisodium citrate and nanominerals on intra-mammary infections rate (IMIR; %) in HF crossbred cows

	<i>Coliforms</i> spp-mediated IMIR (%)		<i>Staphylococcus spp.</i> -mediated IMIR		<i>Streptococcus spp.</i> - mediated IMIR	
	Before (day 0)	After (day 28)	Before (day 0)	After (day 28)	Before (day 0)	After (day 28)
TSC+Nano	12.50	25.00	75.00	50.00	75.00	12.50 (P<0.05)
Healthy	37.50	0	87.50	50	25.00	12.50
SCM	0	66.70	100	100	33.33	50.00

TSC+Nano: SCM affected cows co-administrated with trisodium citrate (TSC) and nanominerals (Nano) (n=8), Healthy cows (n=8), SCM: Subclinical Mastitis (n=6). P<0.05 is statistically significant.

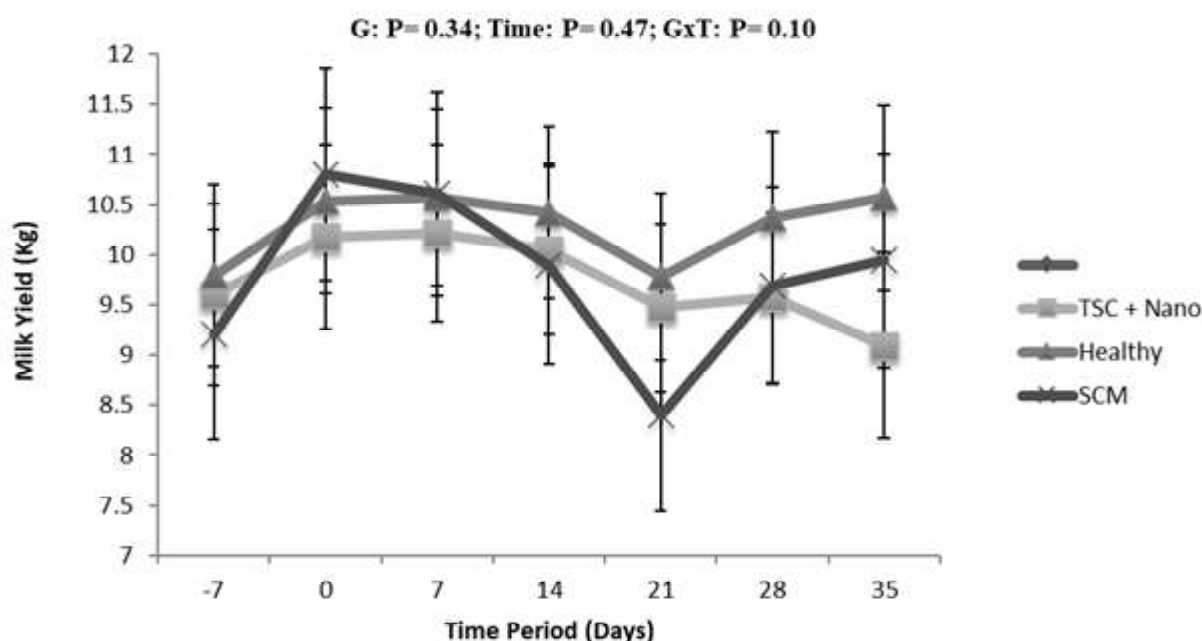


Fig. 1: Effect of co-administration of trisodium citrate (TSC) and nanominerals (Nano) on milk yield (in kg) of subclinical mastitis (SCM) affected cows. Each line represents least square means±SE during pre-treatment (-7 days), treatment (0 to 28 days) and post-treatment period (35 days) in different groups. P<0.05 is statistically significant.

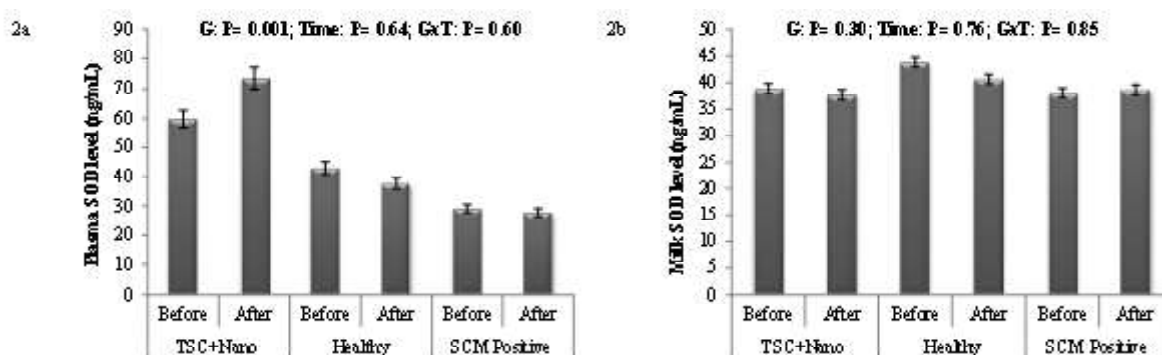


Fig. 2: Effect of co-administration of trisodium citrate (TSC) and nanominerals (Nano) on SOD levels in plasma (Fig. 2a) and milk (Fig. 2b) in SCM affected cows. Each bar represents least square means±SE in different groups during before (day 0) and after (day 28) treatment period. Fixed factors having P<0.05 is statistically significant.

Effect of co-administration of TSC and nanominerals on plasma minerals status and SOD levels in SCM affected cows

Supplementation of Cu and Zn causes no significant changes in their plasma levels. However, plasma Mn levels were significantly increased in supplemented cows compared to non-supplemented cows (G×T: P=0.01) (Table 1). Several studies showed no effects of supplementation on plasma or liver levels. For instance, Cruz et al. (2025) reported that supplementation of Cu, Mn, and Zn from different sources causes no changes in liver mineral status. Cu levels in cows are highly variable and thus the response to

Cu supplementation are also differential (Spearpoint et al. 2025). Similarly, Ganda et al. (2016) reported that supplementation of injectable trace minerals (Cu, Zn, Mn, and Se) in SCM-affected cows causes no significant effect on serum mineral concentrations. In contrast, Williams et al. (2024) reported that nano form of Cu has more bioavailability without any negative effects on health and performance of dairy cows. In this study, co-supplementation of TSC and nanominerals resulted in no changes in plasma (Fig. 2a) or milk SOD levels (Fig. 2b). Silva et al. (2022) reported that Cu, Se, Zn and Mn supplementation in transition dairy cows under heat stress condition causes no

significant effect on antioxidant enzyme levels as observed in this study. Oconitrillo et al. (2024) also reported lack of significant effects of Zn supplementation on antioxidant enzymes and immunoglobulins level in dairy cows. They also reported that despite of higher levels of Zn supplementation, the blood Zn level increased only after 70 days. Simialry, Illek et al. (2023) reported significant increase of milk yield and its composition only after 60 days of organic Zn supplementation. Therefore, duration of supplementation could be an important factor to decide treatment outcome.

Assessing mineral bioavailability through estimation of blood or liver mineral concentrations and minerals-dependent enzyme activity are common methods. But, the relationship between mineral bioavailability and health effects are not clear. For instances, Supplementation of organic Zn reduced milk SCC and improved immunity and antioxidant activity, but no changes was observed in blood Zn level (Klein et al. 2025). Siciliano-Jones et al. (2008) reported improvement in milk yield, hoof health, and fertility with no changes in liver mineral concentrations after supplementation of organic Cu, Zn, and Mn. On the contrary, supplementation improved liver Cu and Zn concentrations but had no effect on mastitis (Nocek et al. 2006). Supplementation of different level and source of Cu, Zn and Mn do not have significant effects on oxidative stress indicators and clinical indices to intramammary LPS challenge in dairy cows (Yasui et al. 2019). Similarly, supplementation of inorganic or organic form of trace minerals (Co, Cu, Mn, Zn and Se) had no changes in Cu, Mn, and Zn levels in serum, liver and urine samples, serum antioxidant activity and incidence of clinical diseases in dairy cows (Mion et al. 2023). Therefore, further studies with an optimal sample size are needed to understand the combined effects of TSC and reduced level of nanominerals in SCM-affected cows, as the sample size of this study is limited.

Conclusions

Altogether, based on the observations such as no changes in milk yield, differential effects on intra-mammary infections rate by major mastitis pathogens and subsequent marginal reduction of udder inflammatory indicators, it is concluded that the co-administration of TSC with half of the recommended levels of nanominerals had no biologically significant effects on recovery of SCM affected cows.

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Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this article.

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