The Pharma Innovation Journal 2022; SP-11(11): 1023-1030

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(11): 1023-1030

www.thepharmajournal.com
Received: 27-09-2022
Accepted: 29-10-2022

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Supplementation of trisodium citrate improves recovery rate of subclinical mastitis in crossbred cows

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Abstract
Mastitis is the top most reason for antibiotic usage, which is an important public health concern due to antimicrobial resistance development in pathogen. Citrate is an important precursor for milk synthesis and regulation of homeostasis between Ca^{2+} and H^{+} ions and thereby milk fluidity. The aim of this study was to evaluate the effect of tribolium citrate (TSC) on recovery of subclinical mastitis (SCM) in HF crossbred cows. Apparently healthy lactating cow's milk samples were screened by somatic cell count (SCC) and California mastitis tests (CMT) methods for three consecutive days. Animals were diagnosed as SCM affected (n=16) or healthy (n=6) using milk SCC cutoff value of 200×10^3 cells/ml (i.e., $\geq 200\times10^3$ cells/ml as SCM and $\leq 200\times10^3$ cells/ml as healthy) and classified into three groups based on parity, stage of lactation and milk yield during pre-treatment period. SCM affected cows (n=8) supplemented with TSC (30 mg/kg B.wt./day for 28 days) and effect of supplementation was evaluated through milk SCC, CMT score, milk yield, milk composition, milk citrate level and bacteriological cure rate in comparison with non-supplemented-SCM affected cows (n=6) and apparently healthy cows (n=8). Data were analyzed by using mixed model and Mc Nemars test. Supplementations of TSC cause no significant changes in milk yield and its composition. Supplementation cause significant ($p < 0.001$) reduction of milk SCC and CMT score with more bacteriological cure rate. It is concluded that supplementation of TSC favored for more bacteriological cure rate in SCM affected cows.

Keywords: Disodium citrate, subclinical mastitis, bacteriological cure rate, california mastitis tests, somatic cell count and crossbred cows

1. Introduction
Mastitis is the most common and costly disease of dairy cattle across the globe including India. Foot and mouth disease and mastitis are major diseases which affects the profitability of Indian dairy farmers (Bardhan, 2013; Varshney and Mukhrjee 2002) [3, 32]. The incidence of subclinical mastitis (SCM) is more common in India compared to clinical mastitis and an estimated annual loss to Indian dairy industry due to mastitis is about 2.37 thousand crore rupees of which, SCM accounted for 70 % of the loss (Varshney and Naresh 2004) [33]. Other reports also estimated more incidence and economic loss due to SCM (58-72 %; 4150-4365 crores) than clinical mastitis (28-42 %; 1700-3000 crores per annum) in India (Dua, 2001; Bansal and Gupta 2009) [11, 2]. Singh *et al*., (2014) [27] reported that the economic losses were more in high yielding crossbred (INR 1, 314.10) than indigenous cows (INR 868.34) and buffaloes (INR 1, 272.36) per lactation. The importance of SCM is due to its more prevalence, it remains as the source of infection for herd mates, its longer duration of infections, it usually proceeds into clinical form, its difficulty of detection and it reduces the milk production and its quality (Tuteja *et al*., 1993; Seegers *et al*., 2003) [31, 25]. Besides, mastitis is the top most reason for antibiotic usage, which is an important public health concern due to antimicrobial resistance development in pathogen (Pol and Reugg 2007) [23].
Citrate is an important precursor for milk synthesis and main component of buffer system in udder for maintenance of normal pH at 6.5, regulation of the homeostasis between Ca^{2+} and H^{+} ions. Citrate is also essential for sequestration of soluble Ca^{2+} in milk and thereby maintenance of milk fluidity (Shennan and Peaker 2000) [26]. Therefore, decrease of milk citrate level cause Ca^{2+} clump formation, which damage udder tissue, induce inflammatory reactions and facilitate for development of mastitis, which in turn cause further decrease of milk citrate level (Dhillon and Singh 2013) [9]. Decrease of milk citrate level in SCM affected cows depends on severity of infection in terms of Na^{+} and Cl^{-} ions changes.

Hence it was hypothesized that supplementation of trisodium citrate (TSC) have beneficial effects in mastitis affected animals and several researchers evaluated the effects of TSC supplementation on mastitis affected dairy animals and found encouraging results (Dhillon *et al*., 1995) [10]. Dhillon *et al*. (1995) [10] administered TSC and found decrease in bacterial count along with raised milk citric acid level and fall of milk pH towards normal level of 6.5. They also found that administration of TSC replenished citrate deficiency and restored milk constituents on acute or sub-acute mastitis affected cows (Singh *et al*., 1997) [28]. The limitations of these studies include inadequate sample size, homogeneity of group before treatment and failures to define the mastitis conditions before and after treatment through standard diagnostic methods.

Milk citrate is synthesized from glucose and acetate in mammary gland and available reports on milk citrate levels are different. Some researchers reported that milk citrate level varies with stage of lactation, milk composition (particularly fatty acid levels), season, intra-mammary infections and diet (Braunschweig and Puhan 1999; Baticz *et al*., 2002) [7, 4] while other reports indicated that mammary epithelium is impermeable to citrate in both directions (Linzell *et al*., 1976) [15]. It indicated that further studies are required to understand the role of citrate in lactating dairy animals. We hypothesized that replenishment of milk citrate levels through supplementation of TSC could enhance the udder health status and restore the deficiency to the normalcy. The aim of this study was to evaluate the effect of TSC on recovery of subclinical mastitis in HF crossbred cows.

2. Materials and Methods

2.1 Study area and experiment animals

The experiment was conducted at Livestock Research Centre (LRC), Southern Regional Station (SRS) of ICAR- NDRI, Bengaluru (Karnataka), India, which is located at an altitude of 920 meter above sea level in the bed of southern plateau and hills agro-climatic zone on 12° 97'N latitude and 77° 56'E longitudes. The climatic condition of the farm is subtropical where the temperature raises up to 36°C in summer and comes down to 15°C in winter season. The average rainfall ranges from 800-1200 annually. About 22 apparently healthy lactating HF crossbred cow's milk samples were screened for udder health status through milk somatic cell count (SCC) and California mastitis test (CMT) tests for three consecutive days. After screening, 14 animals diagnosed with SCM (SCC as reference test using>200×103 cells/ml as a cut off value) and 8 healthy cows (SCC< 200×103 cells/ml) were included and grouped into three groups as follows, based on pre-treatment period parity, stage of lactation and milk yield. Group-1: SCM affected cows (n=8) supplemented with trisodium citrate (TSC) @ 30 mg/kg b.wt/day (M/s Daffodilpharma, Ahmedabad, India) orally in the morning hours in an empty stomach, before milking for 28 days, Group-2: apparently healthy cows (negative control) without any supplementation (n=8), Group-3: SCM affected cows (positive control) without any supplementation (n=6). The experiment was conducted as per the guidelines of Institute of Animal Ethical Committee (IAEC).

All experimental animals were fed as per NRC (2001) [21] recommendation using Institute grown seasonal green fodders (18-22 kg) like Maize, Jowar, Hybrid napier, Parganas, Guinea grass and Cowpea along with Ragi straw (2-3 kg) and commercially available concentrate feed (Nandini gold-cow feed containing 16–18 % crude protein, 70-72 % TDN, 2.5-3.5 % fat, 5.5-6 % crude fibre, 1-1.5 % acid insoluble ash and 10–11% moisture; M/s Karnataka Milk Federation, Bengaluru). The total quantity of 3-5 kg concentrate mixture was divided in equal proportion and fed at the time of milking during morning and evening times, based on milk production of individual cow.

All experimental HF crossbred cows were maintained under loose housing system. The shelter was provided in the centre of the open area and had concrete enforced stone floor with gabled roof, made up of mangalore tiles. The shed was open from all side with watering facility from one side of the paddock. The housing space for cows was provided as per "Bureau of Indian Standard" (BIS). HF crossbred cows were milked twice in a day during 5.00 AM in the morning and 5.00 PM in the evening using bucket type machine milking system. Before application of teat cluster, teats were washed thoroughly and wiped with dry towel. At the end of machine milking, the residual milk was removed by hand milking to ensure complete removal of milk from udder.

2.2 Estimation of milk yield, milk composition and udder health indicators

Daily milk yield (kg) of each cow in three groups (n=22) were recorded by electronic weighing balance individually during both morning and evening milking time in a day and milk yield was recorded before (7 days), during (28 days) and after (14 days) study period. About two to three initials strips of milk were discarded from each quarter and CMT was performed as per manufacturer's recommendation (M/s Immucell Corporation, Portland, USA). About 5 ml pooled milk samples from all functional quarters of each cow were collected in morning milking period for estimation of milk composition and milk SCC. Milk composition (fat, SNF, protein, lactose, ash and freezing point) was estimated by using Milk Analyzer (M/s Vector Automation System, Bengaluru, India) at room temperature. The milk SCC was estimated using a De Laval cell counter (DCC; M/s De Laval, Tuba, Sweden). Cows with a cow composite milk SCC of ≥ 200,000 cells/ml were considered as having SCM, while cows without any abnormality in milk or udder parenchyma, systemic signs and milk SCC of ≤ 200,000 cells/ml were considered as healthy. The SCC values were log converted for analysis.

2.3 Milk culturing

About 10 ml of the pooled milk samples from all four quarters were collected aseptically as per National Mastitis Council guidelines (NMC, 2004) [20] in separate sterile polyethylene screw-capped, wide mouth vials and processed for milk culturing under sterile environment within 2 hrs. Briefly, about one ml of milk (n=22) was added into 9 ml of nutrient broth and incubated at 37 °C for 24 hrs. Loopful of culture positive samples were further inoculated by spread plate method in selective media for *E. coli*, *Streptococcus spp.* and *Staphylococcus spp*. such as Mac Conkey agar, Edwards agar and Mannitol salt agar, respectively, at 37 °C for 24 hrs. All chemicals used in this study were procured from M/s Himedia, Mumbai (India). Bacterial isolates were identified on the basis of morphological characteristics of the colony and gram staining. Number of colonies of each organism was counted and animals were considered as bacteriologically cured or udder infected if no growth and ≥ 10 colonies observed, respectively. Animals were considered as

bacteriologically cured when the bacteria were initially isolated from particular animal milk samples, and showed no growth after supplementation of 28 days. If there was no growth initially but, showed bacterial growth after supplementation or showed new isolates than initial culture, it was considered as new intra-mammary infection.

2.4 Estimation of milk citrate level

Citrate level in milk was estimated by photometric method following the procedure of Ali *et al*., (1998) [1] with little modification. Briefly, an aqueous solution of copper chloride (pH 6.5) was prepared and by adding 1 mg sodium potassium tartrate, the precipitation of cupric ions was prevented. About 10 ml of copper chloride solution was mixed with 1 ml milk sample and 9 ml distilled water in glass tubes separately. All the glass tubes containing solution were kept in water bath at 40 °C for 5 minutes which formed blue colored complex. The absorbance of each sample was taken immediately at 760 nm using UV spectrophotometer (M/s Hitachi).

2.5 Statistical analysis

The findings were analyzed by using mixed-model repeated measure analysis considering group, time and their interaction as fixed effect. Further, the interaction between two groups at each time point of intervals and interaction within a group were analyzed by using univariate method. Bacteriological cure rates were analyzed through non-parametric McNemar test using SPSS version 20 software packages.

3. Results

3.1 Effect of trisodium citrate supplementation on milk yield (in kg) and its composition in SCM affected cows

Daily milk yield was recorded in study animals before (7 days), during (28 days) and after (14 days) experiment period. Fixed factors alone or their interactions (group × time) had no significant effects on average weekly milk yield (Fig.1). Results of interaction of fixed factors (group × time) on milk components such as milk fat (Fig. 2a), protein (Fig. 2b), lactose (Fig. 2c), Solid Not Fat (SNF; Fig. 2d), and ash content (Fig. 2e) were also revealed no significant difference between TSC supplemented and healthy cows during the study period.

3.2 Effect of trisodium citrate supplementation on udder health indicators in SCM affected cows

Group alone had significant (p = 0.001) effects on milk SCC (Fig. 3a) where, SCM affected cows with no supplementation (positive control) had significantly (p = 0.05) increased milk SCC over the time period. Fixed factors alone or interaction of fixed factors had significant effects on CMT score (Fig. 3b). The average CMT score was significantly (p < 0.05) reduced in TSC supplemented group (2.3 Vs 1.8) while, in SCM affected cows with no supplementation had significantly increased CMT score (0.8 Vs 3.0) during study period.

Supplementation of TSC for 28 days resulted in more bacteriological cure rate compared to non-supplemented positive control cows (Table 1). Non-supplemented SCM affected cows suffered with more new intra-mammary infection with coliforms and *Streptococcus spp*. and persistent infection with *Staphylococcus spp*. after the experiment period. Overall, TSC supplementation reduced 25-50 % of intra-mammary infections.

3.3 Effect of trisodium citrate supplementation on milk citrate level in SCM affected cows

Fixed factors alone or their interactions (group × time) had no significant effects on milk citrate levels (Fig. 4).

Table 1: Effect of trisodium citrate supplementation on bacteriological cure rate (%) in crossbred cows

	Mac Conkey agar*		Mannitol salt agar**		Edwards Medium agar#	
	Before	After	Before	After	Before	After
TSC	2/8 (25)	0/8 (0)	5/8 (62.50)	2/8 (25)	6/8 (75)	2/8 (25)
Healthy	3/8 (37.50)	0/8 (0)	7/8 (87.50)	4/8 (50)	2/8 (25)	1/8 (12.5)
SCM	0/6 (0)	4/6 (66.7)	6/6 (100)	6/6 (100)	2/6 (33.33)	3/6 (50)

For selective isolation of *Coliforms, **Staphylococcus sp. and #Streptococcus sp. Figures in parenthesis are percentage. TSC: Trisodium citrate (n=8), Healthy (n=8), SCM: Subclinical Mastitis (n=6). Figures in parenthesis are percentage of intra-mammary infections.

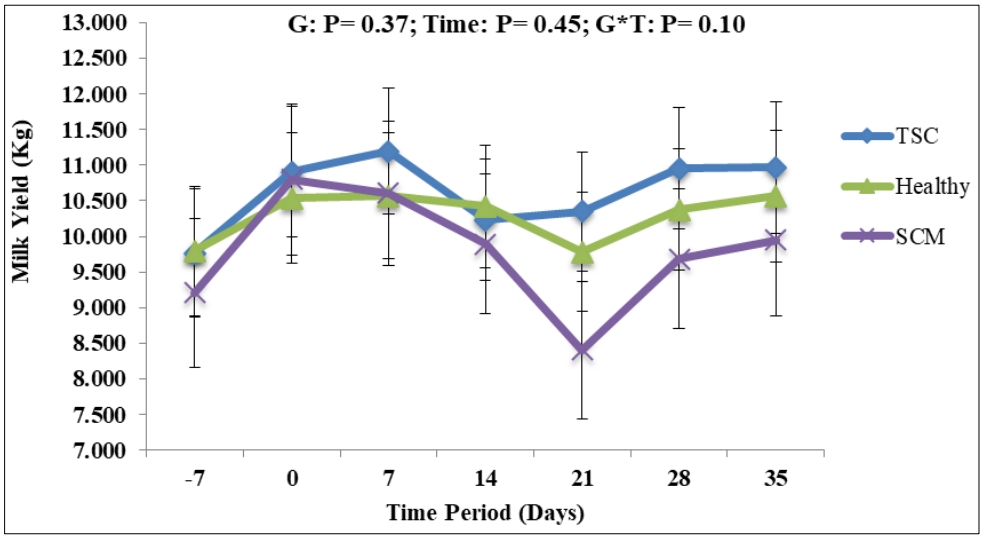
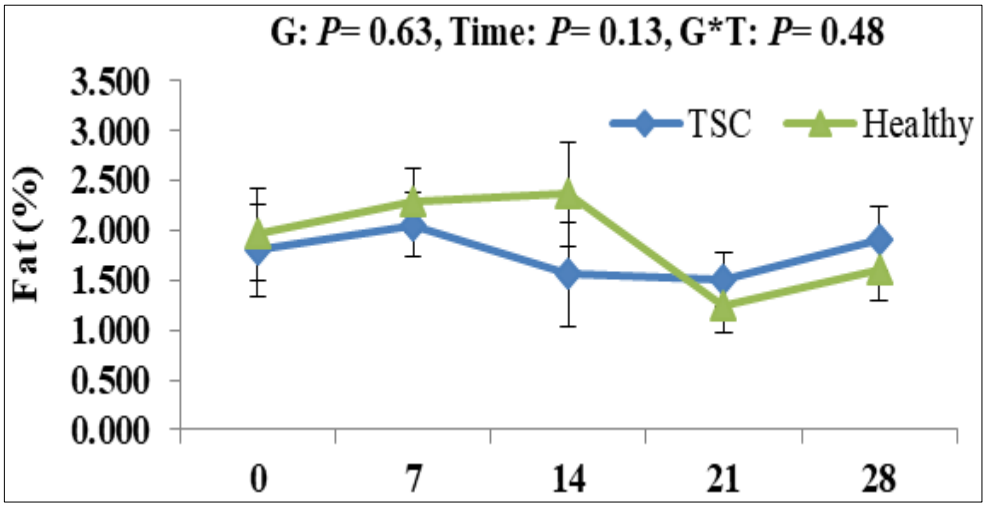
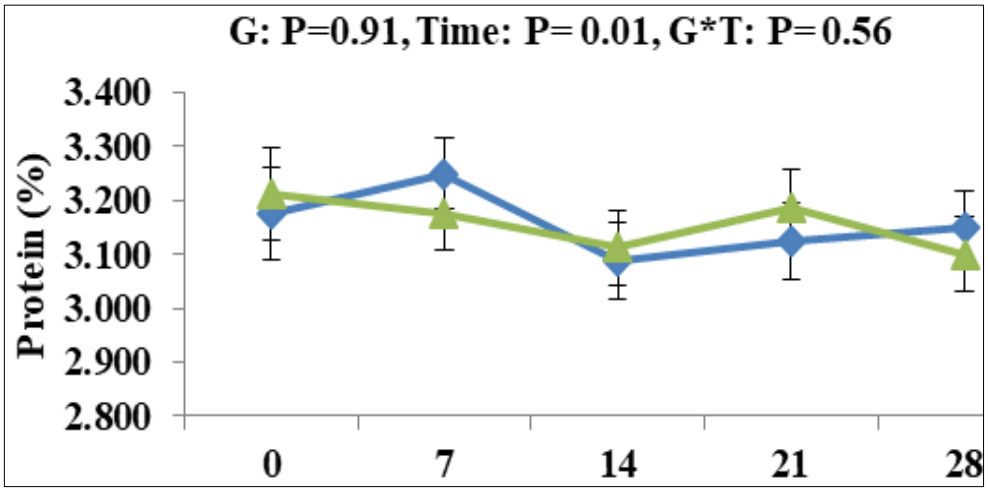


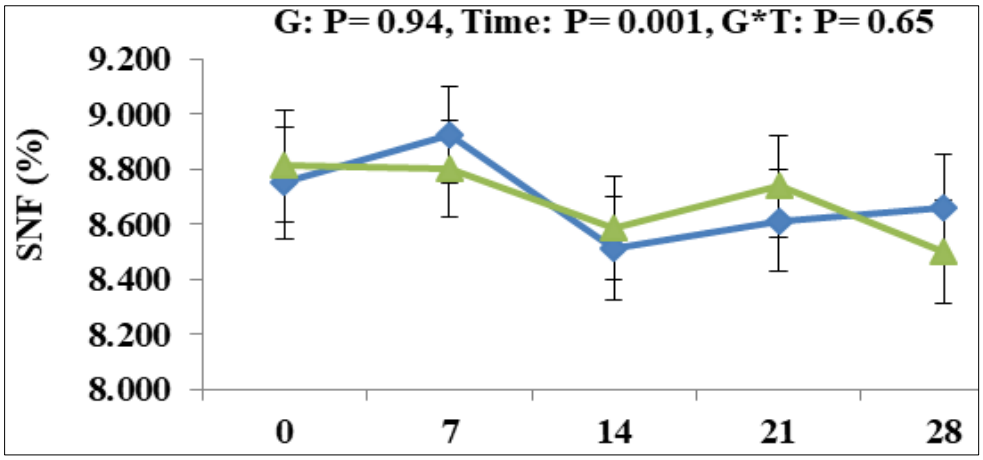
Fig 1: Effect of trisodium citrate (TSC) supplementation on milk yield (kg) in subclinical mastitis (SCM) affected cows. The effects having p < 0.05 are considered as statistically significant


G: P= 0.63, Time: P= 0.13, G*T: P= 0.48
TSC
Healthy
Fat (%)
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0
7
14
21
28

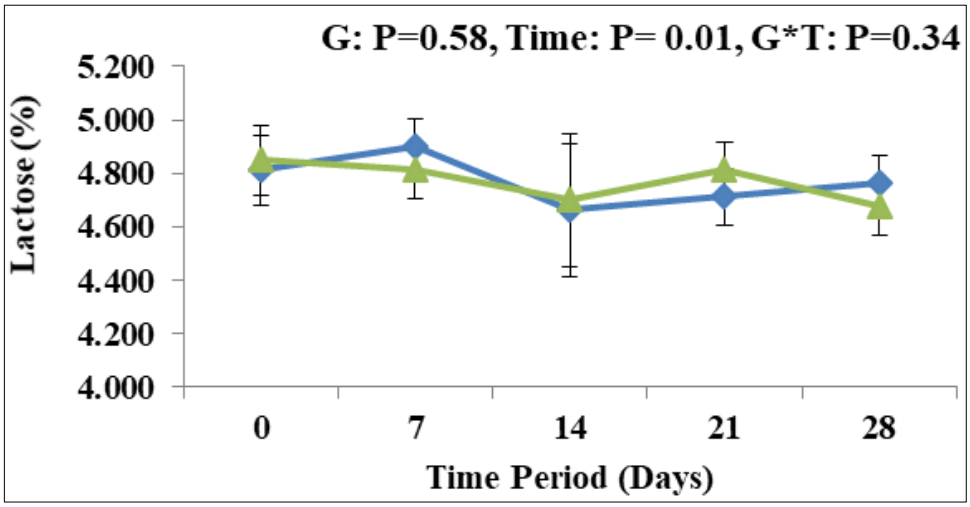
2a


G: P=0.91, Time: P= 0.01, G*T: P= 0.56
Protein (%)
3.400
3.300
3.200
3.100
3.000
2.900
2.800
0
7
14
21
28

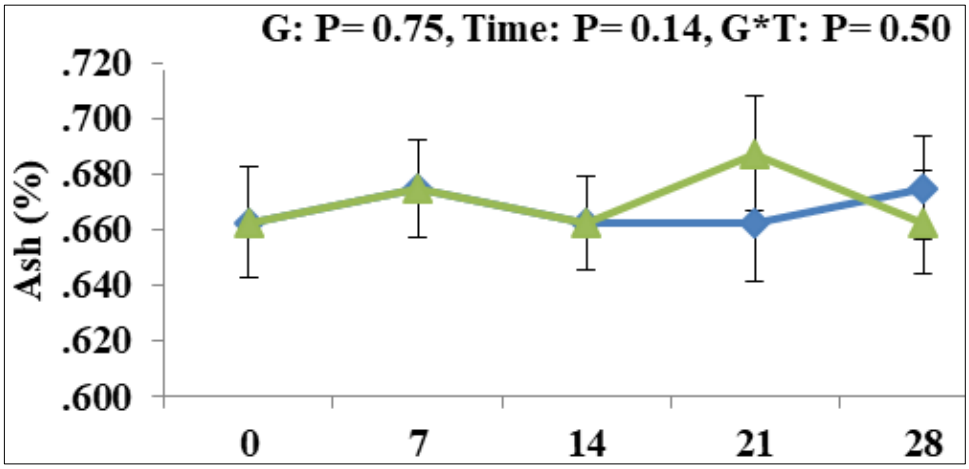
2b


G: P= 0.94, Time: P= 0.001, G*T: P= 0.65
SNF (%)
9.200
9.000
8.800
8.600
8.400
8.200
8.000
0
7
14
21
28

2c



2d



2d

Fig 2: Effect of trisodium citrate (TSC) supplementation on such as Milk fat (Fig. 2a), Protein (Fig.2b), Lactose (Fig. 2c), Solid Not Fat (SNF; Fig. 2d) and Ash (Fig. 2e) content (%) in SCM affected cows. The effects having *P*<0.05 are considered as statistically significant.

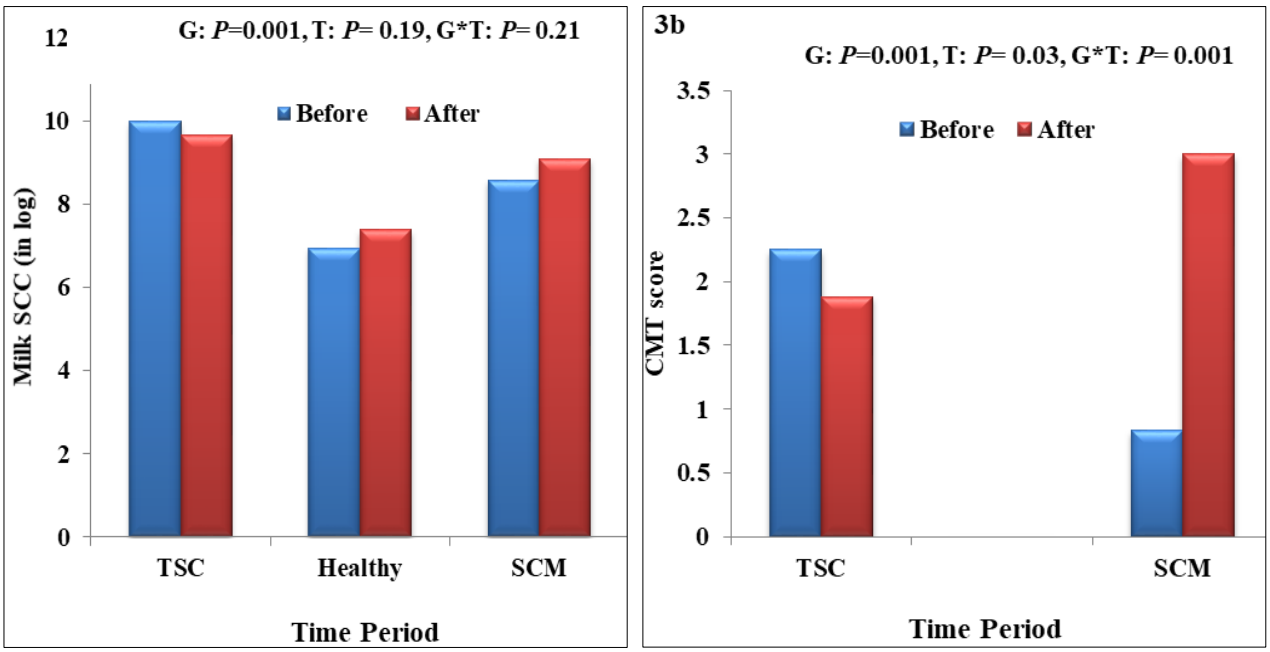


Fig 3: Effect of trisodium citrate (TSC) supplementation for 28 days on udder health indicators such as milk SCC (Fig. 3a; in log; 100× 103 cells/ml) and California Mastitis Test (CMT) score (Fig.3b), before (day 0) and after (day 35) study period in subclinical mastitis (SCM) affected cows. CMT score of healthy cows remains negative (i.e., score 0) during study period. The effects having P<0.05 are considered as statistically significant

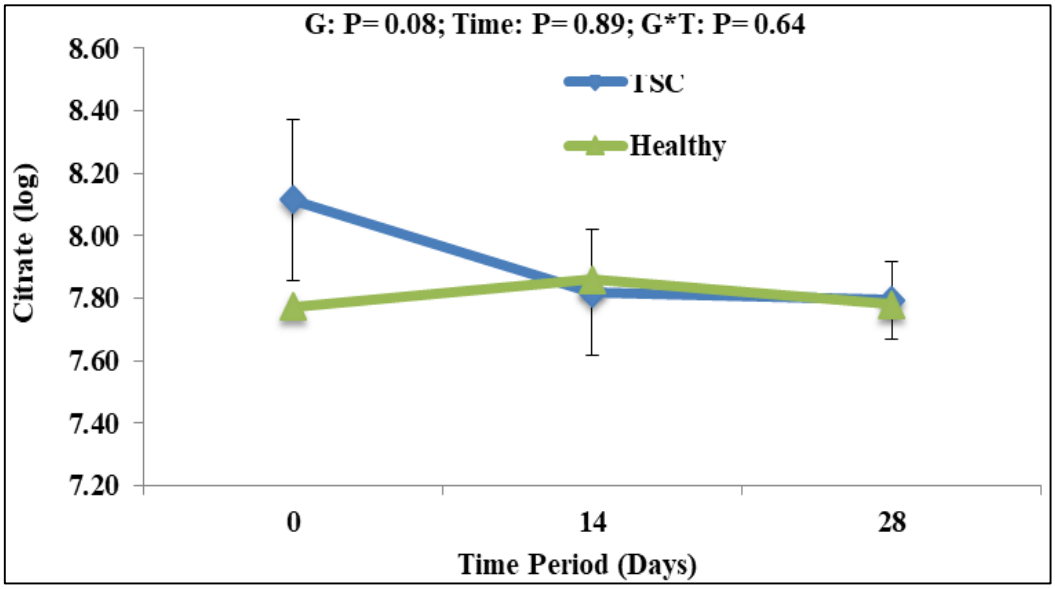


Fig. 4: Effect of trisodium citrate (TSC) supplementation on milk citrate level (log; mg/ 100 ml) in healthy and subclinical mastitis (SCM) affected cows. The effects having $p < 0.05$ are considered as statistically significant

4. Discussion

Fixed factors alone or their interactions had no significant effects on average weekly milk yield but, supplementation of TSC causes marginal increase of milk yield. Several researchers reported that supplementation of TSC had no statistically significant effects on milk yield and its composition as observed in our study (Stumpf *et al*., 2013; Mbonwanayo *et al*., 2016) [30, 16]. Sodium citrate is dissociated into sodium and citrate salt and later ions are subsequently metabolized into bicarbonate ions and released into plasma. Supplementation of bicarbonate buffers in lactating dairy cows also resulted in no changes in milk yield and its fat percentage (Mckinnon *et al*., 1990) [17]. In contrast, combined supplementation of TSC with Vitamin E for four weeks during transition period increased milk yield and fat percentage (Santoshi *et al*., 2018) [24]. Therefore, supplementation of TSC alone may not be beneficial to improve milk yield and combined supplementation of TSC with micronutrient may be beneficial to improve milk yield.

We found non-significant reduction of milk SCC in TSC supplemented SCM cows. Non-significant, marginal reduction of milk SCC after TSC supplementation in mastitis affected cows was also observed in other studies (Stumpf *et al*., 2013; Mbonwanayo *et al*., 2016) [30, 16]. Milk SCC is an accepted indicator of udder health status and thus it is routinely followed for screening in dairy animals. SCC is more of indicator for mammary gland inflammatory reaction rather than actual status of intra-mammary infections. Costa *et al*. (2013) [8] reported that mammary gland takes 14 to 21 days after bacteriological cure, to end the inflammatory reaction and to restore normal condition. Accordingly, non-reduction of milk SCC along with bacteriological cure (as observed in these animals) might be justified in these animals. Non-reduction of milk SCC may also be due to several other factors. For instances, cows with high milk SCC during pre-treatment period, chronically infected animals and rear quarter affected animals had worse response to intra-mammary antimicrobial therapy (Melchior *et al*., 2006; Molina *et al*., 2018) [18, 19], while cows with low SCC experienced more bacteriological cure (Sol *et al*., 2000; Bradley and Green 2009) [29, 6]. In contrast, SCM affected cows with no supplementation (positive control) had significantly ($p < 0.05$) increased milk SCC over the time period, indicating progressive or more new intra-mammary infections as observed in this study. Significant increase of milk SCC in non-supplemented, SCM affected cows and non-significant reduction of milk SCC among TSC supplemented cows indicated that TSC supplementation at least, prevented the further worsening of severity of SCM in cows. The beneficial effect of TSC supplementation in SCM affected cows was also reflected in terms of significant reduction in CMT score ($p < 0.05$) while, the CMT score among SCM affected cows with no supplementation was significantly increased during study period indicating the progressive development of intra-mammary infection in these cows which is very well supported by milk culture findings. Receiver operated characteristics curve analysis revealed 80 % accuracy of CMT in these cows in our earlier study (Halloli *et al*., 2020) [12].

The intra-mammary infection is the presence of an infectious organism in the udder, while SCM implies inflammation of udder, but not necessarily infections. But, SCM mostly associated with bacterial intra-mammary infections (Berry and Meaney 2006) [5]. Bacterial culture based method is the accepted gold standard test for laboratory-based identification of mastitis pathogens (Hogan *et al*., 1999) [13]. In our study, we observed 25-50 % reduction of intra-mammary infections in TSC supplemented SCM cows whereas non-supplemented SCM cows suffered with more new intra-mammary infection. Yousaf *et al*., (2010) [34] also reported 40-45% efficacy in terms of bacteriological count in TSC alone or in combination with levamisole treated SCM affected buffaloes. Dhillon *et al*., (1995) [10] also observed significant reduction in number of colonies across the bacterial group after TSC supplementation in buffaloes, but above studies were not quantified based on absolute bacteriological cure rate as observed in this study. Since the presence of three or more colonies is considered as cultural positive, reduction in few numbers of colonies may not able to reveal the accurate efficacy of bacteriological cure rate. Overall, we observed more bacteriological cure rate of TSC against gram positive than gram negative organisms as also observed by other researchers (Lago *et al*., 2011; Molina *et al*., 2018) [14, 19].

The biological benefits of significantly reduced CMT score, non-significant reduction of milk SCC and more bacteriological cure rate among TSC- supplemented than non-supplemented SCM affected cows, is reflected in terms of marginal increase of average weekly milk yield during study period of 28 days (1.25 kg Vs 0.5 kg). It is noteworthy to mention that the severity of SCM in terms of milk SCC and CMT score during pretreatment period was higher in supplemented cows than non-supplemented cows. Though it is a limitation of this study (availability of lesser number cows in farm), the SCM affected cows able to synthesize marginally more quantity of milk with TSC supplementation during study period, indicating the beneficial effects of TSC. However, the sample size of this study is very less to ensure the effects of TSC on SCM affected cows and thus further studies with optimum sampling size and different dosage are required to understand the therapeutic effects of TSC in SCM affected cows.

The present finding of milk citrate level is in contrast to the previous findings (Dhillon *et al*., 1995; Singh *et al*., 1997) [10, 28] who observed replenishment of milk citric acid after supplementation of TSC in acute and sub-acute mastitis affected dairy animals. But several researchers reported that alteration of milk citrate levels depends on stage of lactation, level of milk production, milk constituents and other non-genetic factors like season and diets (Braunschweig and Puhan 1999; Baticz *et al*., 2002) [7,4]. The milk citrate level is increased during colostrogenesis period of two days before and after calving (Peaker and Linzell 1975) [22]. In this study, uniform distribution of SCM affected and healthy animals before supplementation (average lactation stage of 214 to 248 days and average milk yield of 9.2 to 9.7 kg) and no significant alterations of milk yield and milk composition between supplemented and control groups substantiate the present finding of no alterations of milk citrate levels between supplemented and control cows.

5. Conclusion

Overall, based on the inflammatory indicators and bacteriological cure rate, it is concluded that trisodium citrate supplementations improved udder health status and prevents from development of more severe forms of subclinical mastitis in lactating dairy cows.

6. Acknowledgments

The authors are thankful to Director, ICAR-NDRI, and Head, SRS of ICAR-NDRI for providing needful facilities. Authors are also thankful to in-charge, veterinarian and staffs of LRC, SRS of ICAR-NDRI for their support and assistance.

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