The anti-inflammatory activity of *Taraxacum Mongolicum* in bovine subclinical mastitis and in lipopolysaccharide-stimulated milk cells

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Abstract

To investigate the anti-inflammatory activities of *Taraxacum Mongolicum* (TM) in bovine subclinical mastitis and in lipopolysaccharide (LPS)-stimulated milk cells, fourteen dairy cows with milk somatic cell count (SCC) over 50×10⁴ cells/mL were divided into treatment (n=7) group and control (n=7) group. In the treatment group, each cow was fed with 150 g TM extract daily for 14 days. SCC, fat, protein, lactose, and interleukin (IL)-8 in raw milk was measured before and after the experimental period. In addition, tumor necrosis factor (TNF)-α, IL-1β, IL-8, and nitric oxide (NO) in the supernatant of LPS (1 μg/mL) stimulated, with or without various concentrations of TM extract, milk cells were analyzed. Results showed that cows fed with TM extract significantly (P < 0.05) reduced SCC and IL-8 in milk in comparison with cows in the control group at the end of experiment. Moreover, TM extract inhibited LPS-induced production of TNF-α, IL-1β, IL-8 and NO in milk cells in a dose-dependent manner. These results suggested that the anti-inflammatory effects of TM may be, at least in parts, due to down-regulation of NO and pro-inflammatory cytokines. In conclusion, dietary supplementation of TM extract is a feasible approach to minimize the impact of bovine subclinical mastitis.

Keywords Cytokine, Mastitis, Somatic cell count, *Taraxacum mongolicum*, Traditional Chinese medicine

1. Introduction

Mastitis is one of the most common and costly infectious diseases in the dairy industry, which results in huge economic losses mainly associated with decreased milk production and quality, therapeutic interventions, loss of antibiotic-contaminated milk, and extra labor (Degraves and Fetrow 1993). Mastitis, defined as inflammation of the mammary gland, is usually caused by bacterial infection, and eventually leads to damaged udder tissues (McDougall et al. 2009; Yagi et al. 2002). Mastitis may exist in different forms. Clinical mastitis is an abrupt and severe inflammation of the udder associated with clinical signs. On the other hand, subclinical
mastitis is accompanied with moderate and persistent inflammation without noticeable clinical signs (Jain 1979). Microbiological methods and somatic cell count (SCC) have been used to monitor the health of bovine mammary glands. SCC is a fast and reliable analytical tool reflecting the immunological status of the udder in response to bacterial infections (Leitner et al. 2000). Therefore, SCC is a widely used as an indicator for udder health and milk quality. SCC is composed of lymphocytes, macrophages, polymorphonuclear cells (PMNs), and epithelial cells. The proportion of each subgroup may vary dramatically due to the status of the mammary gland and individual variations (Kehrli and Shuster 1994; Sarikaya et al. 2005). In the milk from healthy udders, macrophages compose of the major cell fraction (Burvenich et al. 1994), play an important role in the course of inflammation through the release of inflammatory mediators, such as nitric oxide (NO) and pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8 (Wittmann et al. 2004). As a consequence, a rapid influx of PMN across the blood/milk barrier occurs, so that PMN become the predominant cell fraction in mastitic milk (Kehrli and Shuster 1994; Sordillo et al. 1997).

In addition, the cell wall components of bacteria, such as endotoxin (lipopolysaccharide, LPS), stimulate the production of pro-inflammatory factors (NO, TNF-α, IL-8, and IL-1β) and may lead to septic shock in severe cases (Boutet et al. 2007). NO is a free radical, derived from the guanidine nitrogen of arginine in a reaction catalyzed by NO synthase (NOS) in various mammalian cells and tissues, plays a significant role in homeostasis and host defense (Xie and Nathan 1994). The large amount of NO produced by NOS-2 has been closely correlated with pathophysiology in a variety of inflammatory diseases (Nathan 1992). When mastitis turns subclinical, there are no noticeable clinical symptoms and can only be diagnosed by microbiology or SCC in milk (Mercer et al. 1976). Subclinical mastitis is also associated with increased IL-8 level in milk, which is interpreted as indicators of the presence of inflammation. The common practice of treating bovine mastitis is intramammary infusion of antibiotics. However, the efficacy is only moderate and the residue of antibiotics in milk has become a public concern (Daley and Hayes 1992). Therefore, using therapeutic agents other than antibiotics for preventing or treating bovine mastitis is an important research subject in the dairy industry.

Plant-based products constitute a major source of alternative therapies for a wide spectrum of diseases in human beings and animals. Although traditional Chinese medicine has been practiced for over 2000 years, the effects of medicinal herbs have not been investigated scientifically until recently. Herbs with anti-inflammatory properties that are used in traditional Chinese medicine, such as Taraxacum mongolicum (TM), may be potential candidates for fulfilling this demand. TM, commonly known as dandelion, belongs to the family Asteraceae and the Taraxcum genus. Dandelions are native to Eurasia and grow in a wide range of temperate regions worldwide. The most common species is Taraxacum officinale (TO), which has long been used in folk medicine to treat hepatic disorders and women’s diseases, such as breast and uterus cancers, and as lactating, choleric, diuretic, and anti-inflammatory remedies (Ahmad et al. 2000; Kisiel and Barszcz 2000; Koo et al. 2004). TO has been shown to significantly decrease carrageenan-induced paw edema (Mascolo et al. 1987; Tita et al. 1993) and the pancreatic weight/body weight ratio in cholecystokinin octapeptide-induced acute pancreatitis in rats (Seo et al. 2005). The leaf extract of TO was able to significantly suppress the production of TNF-α and IL-1β in rat astrocytes stimulated with substance P and LPS (Kim et al. 2000). In this study, the anti-inflammatory potential of TM as an alternative therapy for curing subclinical mastitis was investigated.

2. Materials and methods

2.1. Preparation of TM extract

Taraxacum mongolicum Hand.-Mazz. (herb. No. 421407502; Asteraceae) used for preparing the extract was provided by Koda Pharmaceutics Ltd (Taoyuan, Taiwan). The TM powder was extracted with distilled water in
soxlet apparatus for up to 4 cycles. The extracted material was filtered through sterile muslin cloth and the filtrate was vacuum dried at temperatures below 40°C. The yield was 9.25% w/w with respect to dried and powdered material for TM. The dried powder was weighed and reconstituted in sterile phosphate buffer saline (PBS, pH 7.4, 0.01 M) to a final concentration of 20 mg/ml. The TM solution was filtered through a 0.2-mm filter (Microgen, Laguna Hills, CA, USA), aliquoted, and stored at -20°C until being used.

2.2. Animals
Fourteen Holstein-Friesian cows, between 2 and 7 years of age (1st lactation: n=2, 2nd lactation: n=6, 3rd lactation: n=4, 5th lactation: n=2) and had been lactating for 1 to 8 months, with subclinical mastitis were used in this study. The presence of subclinical mastitis was confirmed by bacteriological analysis and persistently elevated SCC (> 50 × 10⁴ cells/mL). The cows did not receive any treatment during the month prior to the beginning of the experiment. These cows were housed in the animal shed at the farm of Hsin-Chu Branch Station, COA-TLI, Hsin-Chu, Taiwan under identical environmental conditions. The use of animals was approved by the Institutional Animal Care and Use Committee (IACUC).

2.3. Experimental design
The 14 lactating Holstein cows with subclinical mastitis were randomly assigned to 2 groups, control (n=7, basal diet) and treatment (n=7, basal diet with the supplementation of TM herb powder, 150 g per day). Cows were fed daily with total mixed ration, and hay and water were given ad libitum. The TM was added to the concentrates for cows in the treatment group for 14 days (D1 to D14). Milk and blood samples were collected one day before (D0) and one day after (D15) the experimental period.

2.4. Milk sample collection and analysis
Cows were milked twice (5:00 am and 5:00 pm) daily. After cleaning the teat orifice with 70% ethyl alcohol and discarding a few streams of foremilk, milk samples from the four quarters of a cow were collected and mixed (total volume 50 ml). Samples were maintained on ice and shipped to the analytical laboratory immediately. Analysis of milk components (including milk fat, milk protein, lactose) and milk SCC was conducted using a Milk Scan 4000 analyzer (Foss, Hillerd, Denmark) and a Fossmatic 5000 cell counter (Foss, Hillerd, Denmark), respectively.

2.5. Isolation of somatic cells in milk and differential cell counts
Isolation of somatic cells was carried out as described by Sarikaya et al. (2002). Briefly, 1.5 mL of diluted milk sample (30%, vol/vol dilution with cold PBS) was centrifuged for 10 min at 180×g cells. Cells were washed and resuspended in cold PBS (pH7.5). Leukocytes were labeled with SYTO 13 and differential leukocyte count was analyzed by flow cytometry using FACScan (Becton Dickinson) (Dosogne et al. 2003). The numbers of neutrophils, macrophages, and lymphocytes in 10,000 cells were determined and expressed as percentages of the total cell count.

2.6. Culture and treatment of somatic cells in milk
Somatic cells were cultured at 2 × 10⁶/ml in RPMI 1640 medium supplemented with 1% glutamine, 10% fetal calf serum, streptomycin (50 μg/mL), and penicillin (50 IU/mL) (all from Gibco Invitrogen). Cells were cultured at 37°C with or without the presence of LPS (1 μg/mL; Sigma Aldrich, St. Louis, MO) followed by treatments with various concentrations of TM for 24 h before analysis.
2.7. Cell proliferation assay
Cell viability was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Kao et al. 2001). Briefly, cells were cultivated in 25-cm² flasks. After a 24 hr-incubation at 37°C, various amounts of compounds were added to confluent cell monolayers and incubated for another 24 hr. One-tenth volume of MTT (5 mg/mL; Sigma Aldrich, St. Louis, MO) was then added to the culture medium. After 4 hr incubation at 37°C, an equal volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan and the absorbance was measured at 570 nm using a microplate reader.

2.8. Determination of IL-8 levels
Milk and somatic cell in the milk IL-8 levels were determined from undiluted whey samples assayed with a commercially available human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN). The antibody pairs used in this kit have been previously shown to cross-react with bovine IL-8 (Bannerman et al. 2003). The absorbance at 450 nm and a correction wavelength of 550 nm were measured using a microplate reader. Values, expressed in picograms per ml, were extrapolated from a standard curve of known amounts of human IL-8 using linear regression.

2.9. Reverse transcription-polymerase chain reactions
Total RNA was extracted from cells using the RNeasy Mini kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). RT-PCR was performed using the one-step RT-PCR kit (GeneMark, Taiwan). PCR amplification was performed using the primers listed in Table 1. A standard PCR mix was used with 2.5 units of Taq polymerase and supplied buffer (Invitrogen Co., Carlsbad, CA), using 35 cycles of the following steps: 95°C for 45 sec, annealing temperature in Table 1 for 45 sec, 72°C for 1 min and subsequently concluded with 5 min at 72°C. The amplification products were resolved by electrophoresis in 2% agarose gels. Agarose gels were stained with 0.5 mg/mL ethidium bromide in Tris/borate/EDTA buffer (ICN, Costa Mesa, CA).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequences</th>
<th>Predicted Size (bp)</th>
<th>Annealing temperature(°c)</th>
</tr>
</thead>
</table>
| IL-1β    | 5′-CCATGGCAACGTACCTGAACCCA-3′  
5′-AATGGAAACCTCTCTCCCTAAG-3′ | 804 | 55 |
| TNF-α    | 5′-GGACCCCTCAGAAAAAGACACC-3′  
5′-GGGTGCAGGAAGATCTGATCTCTTC-3′ | 702 | 52 |
| β-actin  | 5′-CCAGACAGCAGCTGTGGTGTCG-3′  
5′-GAGAAGCTGTGGCTACGTCGC-3′ | 270 | 55 |

2.10. Nitrate/nitrite concentration
The NO concentration was determined by measuring nitrate and nitrite, the stable end products of NO metabolism, in the culture medium of milk somatic cells. First, nitrate was reduced to nitrite by incubation with nitrate reductase (610 mU/mL) and NADPH (170 mmol/L) at room temperature for 3 hr. Thereafter, 100 μL Griess reagent (Sigma) were added and the optical density at 550 nm (OD 550, corrected for absorbance at 650 nm) was measured by a microplate reader. Nitrite concentrations were calculated from a standard curve of sodium nitrite prepared in PBS. Results were expressed as nmol of nitrate and nitrite (Yeh et al. 2007).
2.11. Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Duncan’s multiple range test was used to determine the significance of differences. A p-value of < 0.05 was considered significant.

3. Results

3.1. TM reduced SCC and IL-8 concentration in milk in vivo

Cows fed with TM had reduced SCC/SCCS (p < 0.05) in milk in comparison with those of cows in the control group (Table 2). The inclusion of TM in the diet also decreased (p < 0.05) the percentage of neutrophils in SCC. In addition, the IL-8 concentration in milk from TM-treated group significantly (p < 0.05) decreased from 410.6 ± 72.5 pg/mL to 165.9 ± 20.9 pg/mL during the 14 days (Fig. 1). Cows fed with TM for 14 days exhibited similar milk composition, including average milk fat, milk protein, and lactose, as that of the control group (Table 3).

Table 2 The statistics of somatic cell count and SCC populations in trial cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment (n=7)</th>
<th>Control (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>SCC (10⁴/ml)</td>
<td>137.6 ± 71.5</td>
<td>61.9 ± 29.0</td>
</tr>
<tr>
<td>SCCS</td>
<td>6.6 ± 0.7</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>3.8 ± 0.2</td>
<td>4.4 ± 2.6</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>53.8 ± 4.2</td>
<td>34.3 ± 15.6</td>
</tr>
<tr>
<td>Macrophage (%)</td>
<td>32.1 ± 7.3</td>
<td>43.5 ± 11.8</td>
</tr>
</tbody>
</table>

Means ± S.E.M. within a column with no common superscripts differ significantly (P < 0.05) as measured by Duncan's multiple range test.

Table 3 The statistics of milk quality in trial cows

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment (n=7)</th>
<th>Average</th>
<th>Control (n=7)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (%)</td>
<td>3.53 ± 0.75</td>
<td>3.54 ± 0.66</td>
<td>3.52 ± 0.21</td>
<td>3.49 ± 0.19</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>3.45 ± 0.31</td>
<td>3.45 ± 0.34</td>
<td>3.45 ± 0.39</td>
<td>3.45 ± 0.34</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>4.72 ± 0.32</td>
<td>4.66 ± 0.24</td>
<td>4.59 ± 0.33</td>
<td>4.64 ± 0.41</td>
</tr>
</tbody>
</table>

3.2. TM had no effects on cell viability in vitro

To assess whether TM affects the cell viability of SCC in milk, MTT assay was performed. In the MTT assay, exposure of various concentrations of TM for 3 days had no significant effects on cell viability (Fig. 2).
Figure 1. The effect of TM on the concentration of IL-8 concentration in milk. After feeding cows a diet supplemented with (n = 7) or without (n = 7) TM for 14 days, milk samples were collected on Day 1 and Day 14. The concentration of IL-8 in milk was determined using an ELISA assay. Data were expressed as the mean ± SEM. * p < 0.05.

Figure 2. The effect of TM on the growth of milk somatic cells. The culture medium was replaced with medium containing various concentrations of TM or medium alone. The number of cells in each culture dish was determined by MTT assay. Values represent the average of duplicate wells from a representative of three independent experiments. The cell number is expressed as $10^4$ cells/well.
3.3. TM decreased LPS-induced upregulation of IL-8, TNF-α, and IL-1β in cultured milk SCC

The anti-inflammatory effects of TM were examined in vitro to elucidate the inhibitory effects on production of inflammatory cytokines in milk SCC. At resting status, the secretion of IL-8 from SCC is low (24.1 ± 21.2 pg/mL) and TM treatment did not increase the secretion of IL-8 (Fig. 3). After LPS stimulation, IL-8 secretion increased to 411.6 ± 34.7 pg/mL and TM treatment suppressed LPS-induced IL-8 secretion in a dose-dependent manner with an IC₅₀ of 73.56 μg/mL. In addition, TNF-α and IL-1β were only minimally expressed in untreated milk cells and the secretion was upregulated in response to LPS (1 μg/mL) stimulation (Fig. 4). TM treatment suppressed the LPS-induced expression of TNF-α and IL-1β mRNA in the milk cells in a dose-dependent fashion.

Figure 3. The effect of TM on the expression of IL-8 in milk somatic cells stimulated by LPS. Confluent somatic cells were stimulated by 1 μg/mL LPS and treated with different concentrations of TM. The concentration of IL-8 after 24 hr was determined using an ELISA assay. Values are expressed as mean ± SEM of 6 experiments. ** p < 0.01. *** p < 0.001.
Fig. 4. Expression of TNF-α and IL-1β in milk somatic cells stimulated by LPS. Milk somatic cells were stimulated by LPS with or without various concentrations (31, 63, and 125 μg/mL) of TM (n = 6). After 24 hr, the cells were collected for mRNA extraction and the expression of TNF-α and IL-1β mRNA was evaluated using RT-PCR. β-actin was used as an internal control for RT-PCR and 28S and 18S were visualized in agarose gels to evaluate the integrity of RNA.

Figure 5. The effect of TM on the concentration of nitrate/nitrite in milk somatic cells. Confluent somatic cells were stimulated by 1 μg/mL LPS and treated with various concentrations of TM. The concentration of nitrate/nitrite in culture medium after 24 hr was determined using an ELISA assay. Values are expressed as mean ± SEM of 6 experiments. ** p < 0.01. *** p < 0.001.
3.4. **TM reduced LPS-induced production of NO in cultured milk SCC**

LPS treatment significantly enhanced the production of NO, as indicated by an increase in nitrite/nitrate (see Fig. 5) of the medium of cultured milk SCC (from 17.4 ± 5.6 nmol to 36.2 ± 2.3 nmol). Nitrite/nitrate in the culture medium of the somatic cells exposed to LPS was significantly suppressed by TM treatment in a dose dependent manner (33.9 ± 4.1 nmol, 31.2 μg/mL; 27.6 ± 2.1 nmol, 62 μg/mL; 26.5 ± 4.4 nmol, 125 μg/mL; 23.7 ± 4.7 nmol, 250 μg/mL; 17.2 ± 1.2 nmol, 500 μg/mL) in comparison to the control.

4. **Discussion**

4.1. **TM reduced SCC and IL-8 concentration in milk in vivo**

To our best knowledge, this is the first study using extract from TM to treat bovine subclinical mastitis. The application of herbal extracts to treat mastitis has been reported previously. Mukherjee et al. (2005; 2010) documented that *Ocimum sanctum* and *Tinospora cordifolia* could enhance the immunity of mammary glands in cows with subclinical mastitis. Intramammary infusion of *Tinospora cordifolia* in cows initially increased milk SCC; however, a significant reduction of milk SCC was observed after 15 days. In our study, results showed that milk SCC and IL-8 in milk were significantly reduced after 14 days of TM treatment in cows with subclinical mastitis. In the milk from healthy udders, macrophages represent the major cell fraction (Paape et al. 1972; Burvenich et al. 1994; Sarikaya et al. 2002), and release pro-inflammatory cytokines (such as TNF-α, IL-1β) and chemokines (such as IL-8) in response to invasion of bacteria (Hoeben et al. 1997; Wittmann et al. 2004; Puneet et al. 2005). As a consequence, a rapid migration of neutrophils from blood into milk occurs (Sordillo and Streicher 2002), so that neutrophils become the predominant cell type in milk from cows with mastitis (Paape et al. 1972; Kehrli and Shuster 1994). Boulanger et al. (2003) also indicated that during the course of subclinical mastitis, the percentage of neutrophils in SCC may increase from 8% to 83%. Immunomediators support the defensive mechanism of mammary glands by exerting potent chemoattractic activities (Persson et al. 1993; Sanchez et al. 1994). Thus, our study revealed that TM treatment particularly reduced (p < 0.05) the number of neutrophils, which is an indicator of alleviation of an established infection.

Moreover, results from the analysis of milk composition before and after TM treatment revealed that dietary supplementation of TM extract for 14 days had no negative effects on milk composition. All the parameters analyzed, including the percentage of protein, fat, and lactose, in milk remained within their normal ranges after TM treatment.

4.2. **TM had no effects on cell viability in vitro**

Schütz et al. (2005) indicated that the toxicity of dandelion was found to be low, due to the absence of significant toxins or alkaloids. TO extracts were demonstrated to exert very low toxicity when administered to rats up to the dose of 10g/kg of body weight (Tita et al., 1993). Akhtar et al. (1985) also reported that rabbits treated orally with dried whole dandelion plants at 3-6 g/kg body weight showed no signs of acute toxicity. In our study, the safety of using TM extract was first examined by culturing various concentrations of TM with milk SCC for up to 3 days. Results indicated that the cell viability of milk SCC was not significantly altered by increasing the concentration of TM extract.
4.3. TM decreased LPS-induced upregulation of IL-8, TNF-α, and IL-1β in cultured milk SCC

The anti-inflammatory effect of TM extract was investigated in vitro by adding various concentrations of TM extract to LPS-stimulated milk SCC. LPS is a potent activator for macrophages and monocytes, which are key mediators of immune responses. Stimulation of macrophages by LPS triggers a cascade of intracellular signaling effects that ultimately leads to expression of cytokines and other inflammatory mediators that constitute the pro-inflammatory responses (Meng and Lowell 1997). Expression of pro-inflammatory enzymes and cytokines is regulated at multiple levels during transcriptional, translational, and posttranslational steps (Vodovotz et al., 1993; Rao, 2000). Production of inflammatory mediators, such as NO and pro-inflammatory cytokines TNF-α, IL-1β, and IL-8, in LPS-stimulated macrophages with or without treatment of TM extract was analyzed. Our results showed that TM extract inhibited the expression of IL-8, TNF-α, and IL-1β in a dose-dependent manner. Many studies have demonstrated that phenolic acids and flavonoids have therapeutic potential against inflammation (Cheng et al., 2004; Leal et al., 2000; Shan et al., 2009). Taraxacum also contains abundant terpenoid and sterol bitter principles (principally taraxacin and taraxacerin), which are equally distributed in the roots, leaves, and flowers. Other terpene/sterol compounds, including beta-amyrin, taraxasterol, taraxterol, and free sterols (sitosterin, stigmasterin, and phytosterin), are structurally related to bile (Leu et al. 2003). Seo et al. (2005) reported the extract of Taraxacum, the major component in the blended herbs, could reduce the concentrations of TNF-α in rats. Koh et al. (2010) also demonstrated the extract of TO leaves inhibited LPS-induced TNF-α and IL-1β production in a dose-dependent manner in RAW 264.7 cells. Thus, the beneficial effect of TM supplementation on subclinical mastitis may also be attributed to the reduced production of pro-inflammatory cytokines.

4.4. TM reduced LPS-induced production of NO in cultured milk SCC

The production of NO in milk SCC stimulated by LPS was significantly reduced by TM extract in a dose-dependent manner as indicated by the Nitrite/nitrate ratio in culture medium. This is in agreement with a previous study indicating that Taraxacum extract was able to inhibit the production of NO and lipid oxidation in vitro (Hu et al. 2005; Koh et al. 2010). Kim et al. (2011) also reported a new eudesmanolide, 1β,3β-dihydroxyeudesman-11(13) isolated from TM had inhibitory effects on NO production in RAW 264.7 cells.

5. Conclusion

Taken together, our results indicated that TM has potent anti-inflammatory effects for treating subclinical mastitis. The mechanism of action may be (i) decreasing the number of infiltrating neutrophils, and (ii) attenuating the expression of TNF-α, IL-1β, IL-8 and NO in milk SCC. This is the first controlled bovine study indicating that dietary supplementation of TM has therapeutic effects on mammary inflammation and serves as an inflammatory mediator through down-regulated expression of pro-inflammatory cytokines and chemokines, so that the infiltration of activated neutrophils and SCC can be reduced. Therefore, TM could be a potential approach for curing bovine subclinical mastitis.

Acknowledgements  This work was supported by the Council of Agriculture, Executive Yuan, Taiwan.
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