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To cite this article: Cuneyt Seckin, Gamze Alpun Kalayci, Nuri Turan, Aysun Yilmaz, Utku Y. Cizmecigil, Ozge Aydin, Juergen A. Richt & Huseyin Yilmaz (2018) Immunomodulatory effects of *Echinacea* and *Pelargonium* on the innate and adoptive immunity in calves, Food and Agricultural Immunology, 29:1, 744-761, DOI: [10.1080/09540105.2018.1444738](https://doi.org/10.1080/09540105.2018.1444738)

To link to this article: <https://doi.org/10.1080/09540105.2018.1444738>



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Published online: 01 Apr 2018.



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Immunomodulatory effects of *Echinacea* and *Pelargonium* on the innate and adoptive immunity in calves

Cuneyt Seckin^a, Gamze Alpun Kalayci^a, Nuri Turan^b, Aysun Yilmaz^b,
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ABSTRACT

Immunomodulatory effects of *Echinacea purpurea* and *Pelargonium sidoides* in calves were investigated. Sixty 25-day-old nonvaccinated calves living in integrated farm unit were randomly selected and were divided into 5 treatment groups consisting of 12 calves each; 4 groups were receiving different amounts and/or times of *Echinacea* and *Pelargonium* and the control group received placebo. Blood levels of γ -interferon, cytokine gene expression, lactoferrin and total IgG were analysed on days 0, 9, and 30. When levels for total IgG, γ -interferon, mRNAs for the γ -interferon, IL-1- β , IL-4 and the tumour necrosis factor- α genes were compared from days 0 to 9 post-treatment, significant differences were found between treated and non-treated calves using various amounts of *Echinacea* and *Pelargonium*; a doses of 250 mg *Echinacea* and 62.5 mg *Pelargonium* for 5 days seems to be ideal. *Echinacea purpurea* and *Pelargonium sidoides* are able to modulate immune functions in calves.

ARTICLE HISTORY

Received 21 December 2017
Accepted 3 February 2018

KEYWORDS

Calf; immunity; cytokine; IgG; γ -interferon; PCR; *Echinacea*; *Pelargonium*

Introduction

Calf health and its management are important for the economy and productivity of cattle operations. Calves are exposed to many stress factors such as transport, inadequate feeding, constant exchange of animals, overcrowding, improper climate and poor hygienic conditions. These stress factors cause a decrease in natural immunity which in turn allows access of pathogens mainly through mucosal sites. Since the calf's immune system is not well established in the first weeks of life, adequate colostral supply and good husbandry are critical to avoid intestinal and respiratory pathogens (Ayrle et al., 2016; Cho & Yoon, 2014; Lorenz et al., 2011). In order to improve calf health and immunity, numerous prophylactic tools such as antibiotics, vaccination, biosecurity and good husbandry are applied (Ayrle et al., 2016; Windeyer et al., 2014; Wu, He, & Kang, 2017).

Phytotherapy is an alternative health tool since it is able to modulate the innate and adaptive immunity with positive results for herd health. In addition, herbal products

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are advantageous to certain antibiotics since they do not promote bacterial resistance (Theisen & Muller, 2012). In order to reduce the antibiotic usage in farm animals, products derived from medicinal plants are used worldwide for the prevention and treatment of infectious diseases in animals and humans. However, the effects of many medicinal herbs on the immune system in animals have not been investigated in detail (Ayrle et al., 2016).

There are several reports on the effects of Echinacea, Pelargonium extracts in the immune system in animals and humans. Among the nine different *Echinacea* species, *Echinacea pallida*, *Echinacea angustifolia* and *Echinacea purpurea* have generally been used as herbal medicine (Zhai et al., 2007a). Various animal studies suggest that *Echinacea* may modulate innate and adaptive immune responses. It has previously been shown that *E. purpurea* activates murine/human macrophages by inducing the production of interferon (IFN) and interleukin (IL)-1 (Rininger, Kickner, Chigurupati, McLean, & Franck, 2000). Echinacin, derived from *E. purpurea*, has been reported to attach to macrophages and T cells, which protects the host from viral invasion (Goel et al., 2002; Xiong, Kadota, Tani, & Namba, 1996). In addition, a polysaccharide derived from *Echinacea* inhibited fungal and bacterial growth in mice and increased the level of reactive oxygen intermediates and macrophage chemotaxis as well as production of IL-10, IL-6, IL-1- β and tumour necrosis factor- α (TNF- α) (Roesler et al., 1991; Steinmuller et al., 1993). However, ethanol extracts of *Echinacea* decreased monocyte and macrophage responses in mice to lipopolysaccharide and endotoxin by downregulating the production of TNF- α and prostaglandin E2 (LaLone et al., 2007; Stevenson et al., 2005). In another study mice, *E. angustifolia* enhanced bacterial killing while *E. pallida* and *E. purpurea* did not have an effect on killing (Zhai et al., 2007b).

Echinacea was found to produce profound effects on immune cells such as an increase in immune cell numbers (Cundell, Matrone, Ratajczak, & Jr, 2003), respiratory cellular activity, lymphocyte activation (Robbers & Tyler, 1999), macrophages, phagocytosis (Goel et al., 2002; O'Neill, McKee, & Clarke, 2002), granulocyte migration (Cundell et al., 2003; O'Neill et al., 2002), natural killer cell cytotoxicity (Agbabiaka, Guo, & Ernst, 2000; Currier & Miller, 2001), interferon (Hayashi, Ohotsuki, Suzuki, & Watanabe, 2001) and cytokine production (Cundell et al., 2003; Robbers & Tyler, 1999) as well as antibody response (Bodinet, Lindequist, Teuscher, & Freudenstein, 2002; Freier et al., 2003; Rehman et al., 1999).

Herbal medicine prepared from the roots of *Pelargonium sidoides* and *Pelargonium reniforme* are traditionally used to treat gastrointestinal and respiratory infections, dysmenorrhoea and hepatic disorders in South Africa (Brendler & van Wyk, 2008). EPs 7630 derived from *Pelargonium* is approved as a medicinal product for the therapy of patients with acute bronchitis, common cold, rhinosinusitis, tonsillopharyngitis or COPD (Agbabiaka et al., 2008; Matthys et al., 2013). In addition, *in vitro* studies showed that *Pelargonium* acts against viral and bacterial infections by inhibiting the attachment to cells of influenza virus, HIV-1, HSV, coronavirus, RSV, parainfluenza virus, EMCV, coxsackie virus, *Streptococcus pyogenes* and *Helicobacter pylori* (Beil & Kilian, 2007; Theisen & Muller, 2012; Helfer et al., 2014; Thale et al., 2011). It has also been reported that EPs 7630 increased the mRNA expression of IL-1- β , IL-6, IL-10, IL-12, IL-18, TNF- α , IFN- α , IFN- γ (Kolodziej & Kiderlen, 2007; Trun, Kiderlen, & Kolodziej, 2006; Thale et al., 2011). However, there is no report on the effect of *Pelargonium* on livestock.

Cytokines play an important role in progression and prevention of infectious diseases by modulating the immune response such as induction of fever, the production of cellular and humoral factors such as antibodies, cytokines, acute phase proteins and interferons. Therefore, it is important to understand cytokine expression levels in immune cells in order to predict the innate and adaptive immune response to different pathogens after infections and vaccinations (Konnai, Usui, Ohashi, & Onuma, 2003; Pacheco et al., 2015; Prohl et al., 2015). The measurement of cytokine expression in blood cells is an important tool and real-time RT-PCR, ELISA, Northern Blotting and flowcytometry are generally used to determine the level of cytokine expression (Brodzki, Kostro, Krakowski, & Marczuk, 2015; Konnai et al., 2003; Pacheco et al., 2015; Prohl et al., 2015). Many studies to measure cytokine expression in humans using real-time RT-PCR was performed (Brendler & van Wyk, 2008; Dapas et al., 2014; Senchina et al., 2006), whereas only a few studies investigated the level of cytokine expression in cattle after administering phytotherapy (Ayrle et al., 2016; Wu, Nardone, & Lacetera, 2009).

Pelargonium sidoides and *Echinacea* extracts are frequently used for the treatment of certain viral and bacterial infections in humans and it has been demonstrated by many researchers that these herbs improve the innate and adaptive immunity. However, the effects of these herbs on calf health and immunity are not fully understood. Therefore, this study was designed to investigate the effects of *Echinacea* and *Pelargonium* on the cytokine gene expression, total IgG, γ -interferon and lactoferrin levels in calves.

Material and methods

Herbal preparation and study animals

In this study, a tablet form of the herbal medicinal product (CEVASTIM Tablet) containing the equivalent quantity of the ampoule formulation of *Echinacea purpurea* (500 mg) and *Pelargonium sidoides* (135 mg) was used orally.

The study consisted of the 25 days old, Holstein, nonvaccinated calves, living in a good husbandry conditions in an integrated farm unit located in the Thrace district of Turkey. Randomly selected calves were divided into 5 animal groups (4 treatment groups and 1 control) consisting of 12 calves in each group. Vaccination status was considered in dividing groups. For this, one control nonvaccinated, one vaccinated ($\frac{1}{2}$ tablet for 5 days), and three nonvaccinated groups ($\frac{1}{2}$ tablet for 5 days, 1 tablet for 5 days, $\frac{1}{2}$ tablet for 10 days) were designed. The vaccinated and nonvaccinated groups received the treatment (CEVASTIM tablet) orally while control receiving placebo (Table 1).

All calves received the same feed and husbandry conditions like the other calves on the farm. The animal groups, amount of product administered, sample collection and study scheme are shown in Table 1. Blood was collected in tubes with EDTA for determination of γ -interferon by ELISA, in TEMPUS Blood RNA tubes (Applied Biosystems, Cat No. 4342792) for evaluation of cytokine gene expression and in serum tubes to obtain sera for Lactoferrin and total IgG analyses as indicated in Table 1.

Determination of γ -interferon, lactoferrin and total IgG levels by ELISA

γ -interferon levels in blood were determined by using a commercial test kit (AniGen TB-feron, Bionote, 3801P0001) as described by the manufacturer. Serum lactoferrin and total

Table 1. Animal groups, amount of product, samples and study scheme used in the study. *Blood for cytokine expression was taken 4 days after stopping oral administration (day 14). **Animals in this group received routine vaccination with a combined viral and bacterial vaccine.

Animal groups/amount of product	Duration of oral administration	Daily administration dose	Days and samples taken		
			Day 0	Day 9	Day 30
½ tablet for 5 days (vaccinated**)	5 days	½ tablet	Blood and sera	Blood	Sera
Control (nonvaccinated)	None	None	Blood and sera	Blood	Sera
½ tablet for 5 days (nonvaccinated)	5 days	½ tablet	Blood and sera	Blood	Sera
1 tablet for 5 days (nonvaccinated)	5 days	1 tablet	Blood and sera	Blood	Sera
½ tablet for 10 days (nonvaccinated)	10 days	½ tablet	Blood and sera	Blood*	Sera

IgG levels were also determined using commercial test kits as described by the manufacturers (BETHYL, Bovine Lactoferrin-ELISA Quantitation Set, E10-126, and (Bovine IgG-ELISA Quantitation Kit, BETHYL, E10-118). All sera were analysed in duplicates.

RNA extraction and reverse transcription

Total RNA was extracted from blood samples by using the Tempus Spin RNA Isolation Kit as described by the manufacturer (Thermo Fisher Scientific, Cat No. 4380204). The amount of RNA in the extracted material (90 µl) was measured using a NanoDrop spectrophotometer (NanoDrop 1000c, Thermo Scientific, Waltham, MA, USA). The extracted RNA (about 300 ng) was used for cDNA synthesis.

Reverse transcription and generation of cDNA were performed with 10 µl RNA using a high-capacity Reverse Transcription Kit (ThermoFischer Scientific, Cat No: 4368813). Twenty (20) microlitre of cDNA was generated according to the manufacturer's instruction.

SYBR-green real-time RT-PCR assay for β-actin and cytokine gene expression

The method used for β-actin, γ-interferon and IL-1-β gene expression analyses was a modification of the method described previously (Prohl et al., 2015). The optimized SYBR-green real-time RT-PCR consisted of a total volume of 25 µl reaction mixture, containing (i) 12.5 µl Power SYBR-Green PCR Master Mix (Applied biosystems ThermoFisher Scientific, Cat No. 4367659), 0.4 µl F primer (10 pMol/µl), 0.4 µl R primer (10 pMol/µl), 0.25 µl MgCl₂ (25 mMol), 9.45 µl nuclease free water and 2 µl cDNA for β-actin as the housekeeping gene, (ii) 12.5 µl Power SYBR-Green PCR Master Mix (Applied biosystems ThermoFisher Scientific, Cat No. 4367659), 1 µl F primer (100 pMol/µl), 1 µl R primer (100 pMol/µl), 0.5 µl MgCl₂ (25 mMol), 8 µl nuclease free water and 2 µl cDNA for γ-interferon and (iii) 12.5 µl Power SYBR-Green PCR Master Mix (Applied biosystems ThermoFisher Scientific, Cat No. 4367659), 2 µl F primer (10 pMol/µl), 2 µl R primer (10 pMol/µl), 0.5 µl MgCl₂ (25 mMol), 6 µl nuclease free water and 2 µl cDNA for IL-1-β. The mixture was placed in a thermal cycler (Stratagene

Mx3005P) and the polymerase activated, by incubation at 95°C for 10 min. Cycling conditions were 95°C for 15 s, 63°C for 20 s and 72°C for 15 s for 45 cycles. In order to determine the melting curve, the thermal cycler was programmed to read the fluorescence from 60°C to 99°C in 1°C increments every 10 s.

For IL-2 and IL-4, an optimized SYBR-green real-time RT-PCR reaction was performed by a modified protocol described previously (Konnai et al., 2003). Total volume of 25 µl reaction mixture containing (i) 12.5 µl Power SYBR-Green PCR Master Mix (Applied biosystems Thermo Fisher Scientific, Cat No. 4367659), 1 µl F primer (20 pMol/µl), 1 µl R primer (20 pMol/µl), 0.5 µl MgCl₂ (25 mMol), 8 µl nuclease free water and 2 µl cDNA for IL-2 and (ii) 12.5 µl Power SYBR-Green PCR Master Mix (Applied biosystems ThermoFisher Scientific, Cat No. 4367659), 0.4 µl F primer (20 pMol/µl), 0.4 µl R primer (20 pMol/µl), 9.7 µl nuclease free water and 2 µl cDNA for IL-4. Cycling conditions were 94°C for 1 s, 52°C for 5 s and 72°C for 10 s for 50 cycles. In order to determine the melting curve, the thermal cycler was programmed to read the fluorescence from 60°C to 99°C in 1°C increments every 10 s.

Data and statistical analyses

For the gene expressions study, to control suitability of selected internal control gene, PCR efficiency was calculated for cytokine genes and β-actin gene by tenfold serial dilution of cDNA. A plot was generated with the C_T (*y*-axis) versus log cDNA dilution (*x*-axis) and slope was determined. PCR efficiency (E) was calculated by the equation slope = $-(1/\text{Log}E)$. The mRNA expression was analysed triplicate for each cytokine genes and β-actin gene. Delta C_T (ΔC_T) was calculated between each cytokine gene and β-actin gene and fold changes between treated and control groups was calculated using $2^{-\Delta\Delta C_T}$ formula (Schmittgen & Livak, 2008). The *P*-value (*P* < .05) were determined based on Student's *t*-test for checking internal control gene (β-actine) changes under the experimental conditions and between ΔC_T values for control and treatment groups. Student's *t*-test was performed using GraphPad Prism version 7.00 (Yuan, Reed, Chen, & Stewart Jr, 2006).

Results

γ-interferon, lactoferrin and total IgG levels

γ-interferon levels

γ-interferon levels varied amongst experimental and control animals. The highest Optical Density (OD) obtained after day 9 was 1500 (Figure 1(A,B)). The increase in γ-interferon levels on day 9 was statistically significant in groups received ½ tablet for 5 days vaccinated (ID 1–10), ½ tablet for 5 days (ID 21–30) and ½ tablet for 10 days (ID 41–50) (Figure 1(A,B)).

Lactoferrin levels

Lactoferrin levels varied amongst experimental and control animals. The highest OD obtained after day 9 was 1590 (Figure 2(A,B)). The increase in lactoferrin levels from days 0 to 9 in all treatment groups was not statistically significant. However, a decrease seen in animals ½ tablet administered for 5 days (ID 21–30) was statistically significant.

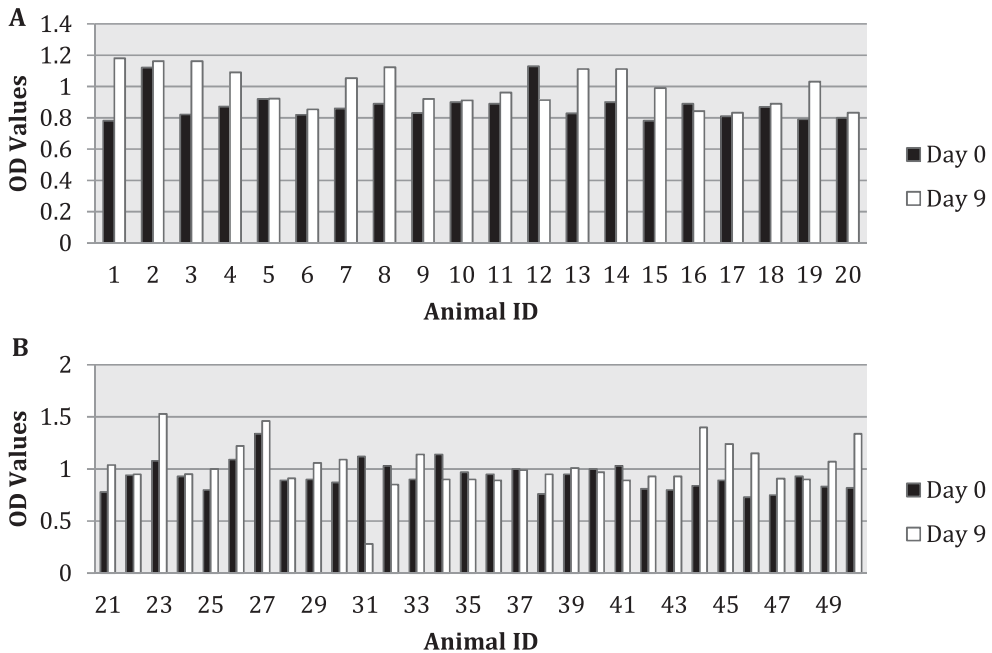


Figure 1. (A,B) OD of γ -interferon levels of all groups before (day 0) and after (day 9) oral administration. Animal ID: from 1 to 10 – $\frac{1}{2}$ tablet for 5 days vaccinated; from 11 to 20 – controls; from 21 to 30 – $\frac{1}{2}$ tablet for 5 days; from 31 to 40 – 1 tablet for 5 days; from 41 to 50 – $\frac{1}{2}$ tablet for 10 days. Light bars: day 0; dark bars: day 9.

Total IgG levels

Total IgG levels varied amongst experimental and control animals. The highest OD obtained after day 9 was 0.900 (Figure 3(A,B)). The difference in total IgG levels between the days 0 and 9 was statistically significant only in animals given $\frac{1}{2}$ tablets for 5 days (ID 21–30; Figure 3(A,B)). There was an increase in the total IgG level in animals received $\frac{1}{2}$ tablet for 5 days vaccinated (ID 1–10), but this was statistically not significant ($P = .08$).

β -actin and cytokine gene expression

PCR efficiency of the β -actin and cytokine genes were found to be between 1.8 and 2.2 which is sufficient for comparison. The mean and 95% of confidence intervals of fold changes for all cytokine genes are given in Table 2 and Figure 4. Results of the statistical analyses of the delta C_T values between reference and target (cytokine) genes for both control and treatment groups are given in Table 3. There were no significant changes in β -actin gene expression before (day 0) and after treatment (day 9) except the group of animals received $\frac{1}{2}$ tablet treatment for 10 days (Table 3). The results of the animal groups in target gene expression are explained below.

Animal treatment groups for cytokine gene expression

$\frac{1}{2}$ tablet 5 days-vaccinated group

Interferon γ , IL-1 β and IL-2, IL-4 and TNF- α gene expression were upregulated in the animals treated with $\frac{1}{2}$ tablet product for 5 days and received routine vaccination

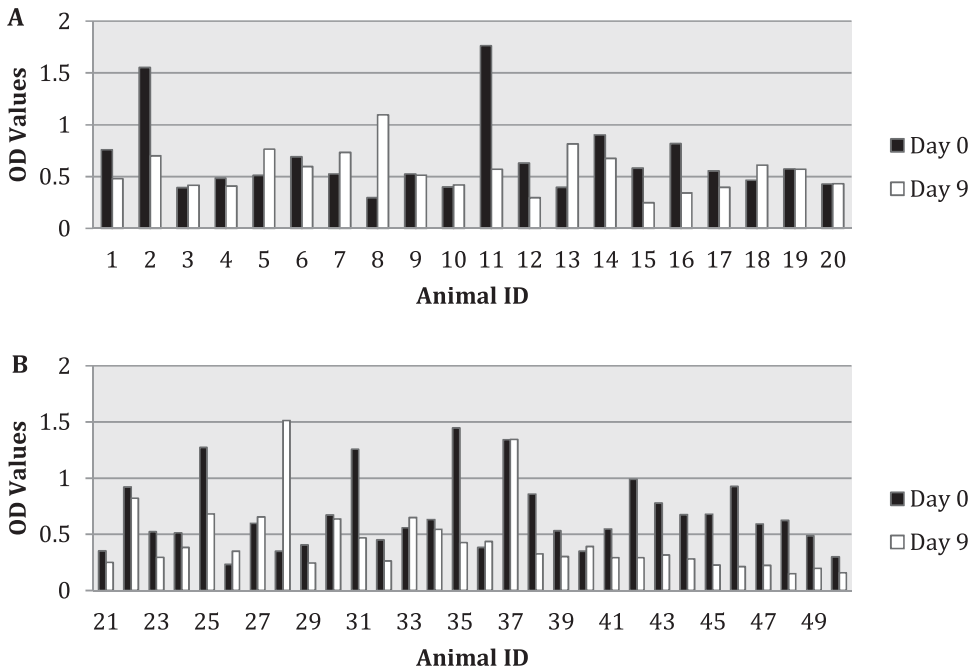


Figure 2. (A,B) OD of Lactoferrin levels of all groups before (day 0) and after (day 9) oral administration. Animal ID: from 1 to 10 – ½ tablet for 5 days vaccinated; from 11 to 20 – controls; from 21 to 30 – ½ tablet for 5 days; from 31 to 40 – 1 tablet for 5 days; from 41 to 50 – ½ tablet for 10 days. Light bars: day 0; dark bars: day 9.

(Table 2, Figure 4). The gene fold expression calculated by using the values in control (day 0) and after (day 9) treatment for interferon γ , IL-1- β , IL-2 and TNF- α were statistically significant (Table 3). Changes in control groups were not statistically significant (Table 3).

½ tablet for 5 days-nonvaccinated group

Interferon γ , IL-1- β and IL-2, IL-4 and TNF- α gene expression were upregulated in the animals treated with ½ tablet product for 5 days (Table 2, Figure 4). The gene fold expression calculated by using the values in control (day 0) and after (day 9) treatment for interferon γ , IL-1- β , IL-2 and TNF- α were statistically significant (Table 3). Changes in control groups were not statistically significant (Table 3).

1 tablet for 5 days-nonvaccinated group

Interferon γ , IL-1- β and IL-2, IL-4 and TNF- α gene expression were upregulated in the animals treated with 1 tablet product for 5 days (Table 2, Figure 4). The gene fold expression calculated by using the values in control (day 0) and after (day 9) treatment for IL-1- β , IL-2 and TNF- α were statistically significant (Table 3). Changes in control groups were not statistically significant (Table 3).

½ tablet for 10 days-nonvaccinated group

Interferon γ , IL-1- β and IL-2, IL-4 and TNF- α gene expression were upregulated in the animals treated with 1 tablet product for 5 days (Table 2, Figure 4). The gene fold

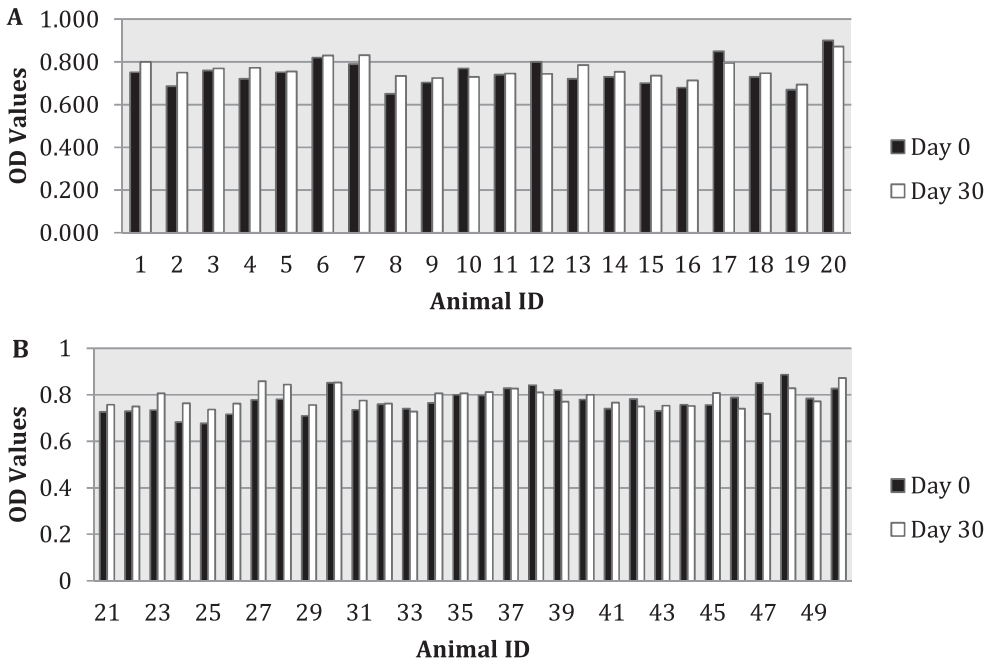


Figure 3. (A,B) OD of total IgG levels of all groups before (day 0) and after (day 9) oral administration. Animal ID: from 1 to 10 – ½ tablet for 5 days vaccinated; from 11 to 20 – controls; from 21 to 30 – ½ tablet for 5 days; from 31 to 40 – 1 tablet for 5 days; from 41 to 50 – ½ tablet for 10 days. Light bars: day 0; dark bars: day 9.

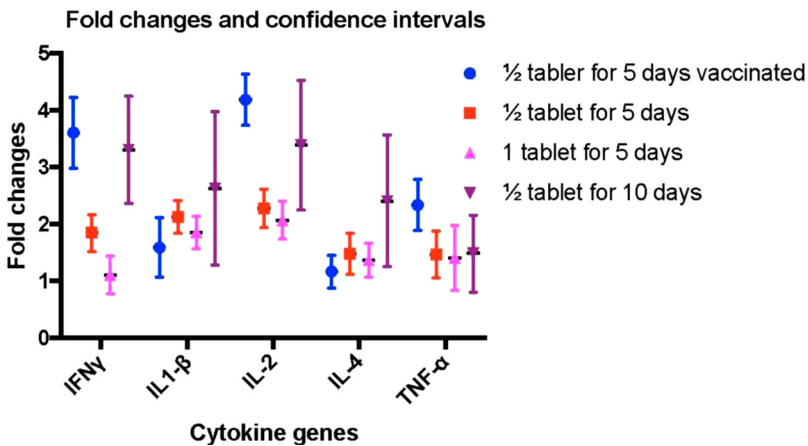


Figure 4. The mean and 95% of confidence intervals of fold changes for all cytokine genes in animal treatment groups.

expression calculated by using the values in control (day 0) and after (day 9) treatment for interferon γ , IL-1- β , IL-2, IL-4 and TNF- α was statistically significant (Table 3). Changes in control groups were also statistically significant (Table 3). Therefore, the upregulation in the treated group cannot be concluded as upregulation.

Table 2. The mean and 95% of confidence intervals of fold changes for all cytokine genes in animal treatment groups.

Animal treatment groups	γ -interferon	γ -interferon %95 CI	IL-1- β	IL-1- β %95 CI	IL-2	IL-2 %95 CI	IL-4	IL-4 %95 CI	TNF- α	TNF- α %95 CI
½ tablet for 5 days (vaccinated)	3.61	2.98–4.23	1.59	1.07–2.12	4.19	3.74–4.64	1.17	0.88–1.45	2.34	1.89–2.79
½ tablet for 5 days (nonvaccinated)	1.85	1.52–2.17	2.13	1.84–2.42	2.28	1.94–2.62	1.48	1.12–1.84	1.47	1.06–1.88
1 tablet for 5 days (nonvaccinated)	1.11	0.78–1.44	1.86	1.57–2.14	2.07	1.74–2.41	1.37	1.07–1.67	1.41	0.84–1.98
½ tablet for 10 days (nonvaccinated)	3.31	2.37–4.25	2.63	1.28–3.98	3.39	2.25–4.53	2.41	1.25–3.57	1.49	0.81–2.16

Table 3. Summary of the results from the statistical analyses of the delta C_T values between reference and cytokine genes for both control and treatment groups. NS: statistically not significant; S: statistically significant ($P < .05$).

Animal treatment groups	γ -interferon	IL-1- β	IL-2	IL-4	TNF- α	Over all β -actine gene variation under treatment
½ tablet for 5 days (vaccinated)	S (P .0094)	S (P .0312)	S (P .0007)	NS	S (P .0031)	NS
Control (nonvaccinated)	NS	NS	NS	NS	NS	
½ tablet for 5 days (nonvaccinated)	S (P .0015)	S (P .0474)	S (P .0339)	NS	S (P .0056)	NS
1 tablet for 5 days (nonvaccinated)	NS	S (P .0464)	S (P .0138)	NS	S (P .0096)	NS
½ tablet for 10 days (nonvaccinated)	S (P .0002)	S (P .0028)	S (P .0358)	S (P <.0001)	S (P .0304)	S

Table 4. Summary of the results from ELISA for γ -interferon, lactoferrin and total IgG levels in all groups. NS: statistically not significant; S: statistically significant ($P < .05$).

Animal treatment groups	γ -interferon ELISA	IgG-ELISA	Lactoferrin-ELISA
½ tablet for 5 days (vaccinated)	S	NS (P = .08)	NS
Control (nonvaccinated)	NS	NS	NS
½ tablet 5 days (nonvaccinated)	S	S	NS
1 tablet 5 gün (nonvaccinated)	NS	NS	NS
½ tablet 10 gün (nonvaccinated)	S	NS	S (Decrease)

Summary of the comparison of all groups for all parameters analysed

Summary of the statistically significance and nonsignificance for all parameters are shown in Tables 3 and 4.

The increase in γ -interferon levels on day 9 was statistically significant in groups received ½ tablet for 5 days-vaccinated, ½ tablet for 5 days and ½ tablet for 10 days (Table 4). The difference in total IgG levels between the days 0 and 9 was statistically significant only in animals given ½ tablets for 5 days (Table 4). However, lactoferrin levels were decreased in all groups (Table 4).

For the cytokine gene expression; in ½ tablet 5 days-vaccinated group, the gene fold expression for interferon γ , IL-1- β , IL-2 and TNF- α was statistically significant (Table 3). In ½ tablet for 5 days-nonvaccinated group, the gene fold expression for interferon γ , IL-1- β , IL-2 and TNF- α was statistically significant (Table 3). In 1 tablet for 5 days-nonvaccinated group, the gene fold expression for IL-1- β , IL-2 and TNF- α was statistically significant (Table 3). In ½ tablet for 10 days-nonvaccinated group, the gene fold expression for interferon γ , IL-1- β , IL-2, IL-4 and TNF- α was statistically significant in both study group and control group (Table 3). Therefore, the upregulation in this group cannot be calculated and therefore cannot be considered to be as upregulation.

Discussion

Respiratory and mucosal pathogens have a significant impact on the cattle health (Cho & Yoon, 2014; Pfützner & Ozsvári, 2017; Windeyer et al., 2014). Neonatal calf diarrhoea and respiratory infections are found to be the most frequent cause of calf losses with a mortality up to 55% in many countries (Ayrle et al., 2016; Bähler et al., 2012; Cho & Yoon,

2014; Lorenz et al., 2011; Meganck, Hoflack, & Opsomer, 2014; Windeyer et al., 2014). Vaccines, antibiotics, biosecurity and good husbandry are generally applied to combat those infections in calves. In addition to these, alternative approaches like herbal products are needed to promote calf immune system to combat pathogens in calves. In contrast to human and experimental animals, there are not many studies related to use of phytotherapy to investigate immunomodulatory effect in farm animals (Basmacıoğlu Malayoğlu et al., 2010; Ayrle et al., 2016; Baruh & Kacabağlı, 2017). In the absence of comprehensive information on the effect of *Echinacea* and *Pelargonium* in cattle immunity, this study was performed to investigate potential efficacy of *Echinacea* and *Pelargonium* on the cytokine expression, total IgG, γ -interferon and lactoferrin levels in calves.

Up to date, the effect of *Echinacea*, Ginseng and *Tinospora* on the immune modulation of cattle to pathogens have been investigated (Hu, Concha, Lin, & Persson Waller, 2003; Mukherjee, De, & Ram, 2010; Wu et al., 2009). In one study, extract of *Echinacea angustifolia* increased the PBMNC count and interferon production in cattle (Wu et al., 2009). In another study, using the Ginseng extract in cattle with mastitis has formed an adjuvant effect when combined with ovalbumin and *Staphylococcus aureus* bacterin by increasing lymphocyte count and the antibody response (Hu et al., 2003). In the other study, in cattle with mastitis, the extract of *Tinospora cordifolia* has increased the somatic cell count, phagocytic and lysosomal enzyme activity and IL-8 level, and caused reduction of the total bacterial count in milk (Mukherjee et al., 2010). Results of these studies indicated that medicinal herbs have potential to modulate immune system in cattle.

In the present study, using the *Echinacea* and *Pelargonium* extract in calves have increased total IgG level, γ -interferon level and cytokine gene expression. *Echinacea* also caused an increase in immunological parameters in dogs and sheep. In dogs which received *Echinacea* formulation, PCV, Hb, RBC count, WBC count, Lym, Nut, the per cent of phagocytosis and IgM level were significantly increased ($P < .05$) (Torkan, Khamesipour, & Katsande, 2015). In sheep, *Salmonella enteritidis* (SE) bacterin and dried roots of *Echinacea purpurea* (EP) were used together and enhancement in antibody production was reported (Barbour et al., 2015).

Species of an herb, the amount of the product, duration of administration and the method analysed will effect the immunomodulation of the medicinal herbs in a given animal (Ayrle et al., 2016). In the present study, *Echinacea* and *Pelargonium* were used since the results of previous studies have shown that these herbs modulate the immune system (Ayrle et al., 2016; Barrett, 2003; Brendler & van Wyk, 2008; Kayser, Kolodziej, & Kiderlen, 2001). The amount and duration of these herbs were tested in this study by using a different amount of CEVASTIM in different durations. The method, real-time RT-PCR to estimate cytokine expression levels used in this study was also used by many investigators (Dapas et al., 2014; Konnai et al., 2003; Puech, Dedieu, Chantal, & Rodrigues, 2015). Results of previous studies have indicated that cytokine gene expression usually increases after 72–96 hours of immunization (Dar, Hajam, Suryanarayana, Kishore, & Kondabattula, 2015). Therefore, in the present study, blood was taken to measure cytokine gene expression level 96 hours after stopping oral administration of CEVASTIM.

In contrast to calves, immunomodulatory effect of *Echinacea* and *Pelargonium* has been widely investigated in human and experimental animals (Agbabiaka et al., 2008; Moyo & Van Staden, 2014; Uluisık & Keskin, 2012; Wagner et al., 2015). It has been demonstrated

that extract of both plants especially *Pelargonium* extract (EPs 7630) have anti-infective properties (Brendler & van Wyk, 2008; Conrad & Frank, 2008; Timmer et al., 2013). In a study performed by Hall and others (2007), *Echinacea purpurea* reduced the duration of upper respiratory infections in human. In influenza vaccinated human, four *Echinacea* species (*E. angustifolia*, *E. purpurea*, *E. simulata*, *E. tennesseensis*) had no effect on IFN- γ production, diminished the IL-2 production but augmented the IL-10 production. However, *Echinacea pallida* suppressed expression of all cytokines (Senchina et al., 2006). In the present study, in treatment groups of animals, *Echinacea* and *Pelargonium* combination had no effect on IL-4 gene upregulation but upregulated the other targeted cytokine genes except the group which received $\frac{1}{2}$ tablet for 10 days. In one study, *Echinacea* was found to have anti-inflammatory properties since reduced severity of symptoms have been seen *in vivo* during the treatment of upper respiratory tract infections with *Echinacea* (Fast, Balles, Scholten, Mulder, & Rana, 2015). However, TNF- α production was decreased. In the present study, the TNF- α gene expression was upregulated in 3 treated groups ($\frac{1}{2}$ tablet for 5 days vaccinated, $\frac{1}{2}$ tablet for 5 days and 1 tablet for 5 days) when compared to controls. In another study in human, a syrup containing Echinacoside and polysaccharide from *Echinacea* was administered to 10 people once a day for 1 month. Results showed that IL-2 and IL-8 were upregulated while the mRNA levels of the pro-inflammatory cytokines TNF- α and IL6 downregulated. This regulation was correlated with the cytokine levels in plasma (Dapas et al., 2014). In the present study, similar results were obtained that IL-2 expression was upregulated except in a group of animals received $\frac{1}{2}$ tablet products for 10 days. In one study, the efficacy of 5 days of *Pelargonium sidoides* therapy for preventing asthma attack during upper respiratory tract viral infections was investigated. Sixty-one asthmatic children with upper respiratory tract viral infection were enrolled in the study which indicated the children administered *Pelargonium sidoides*, had less frequency of asthma attack (Tahan and Yaman, 2013). Similarly, in the present study, the 5 days regime had a better effect on targeted cytokine gene expression in calves except IL-4. In another study in human, EPs[®] 7630 (100 μ g/ml) interfered with replication of seasonal influenza A virus strains (H1N1, H3N2), human coronavirus, respiratory syncytial virus, parainfluenza virus, and coxsackie virus but did not have any effect on the replication of highly pathogenic avian influenza A virus (H5N1), rhinovirus or adenovirus (Michaelis, Doerr, & Cinatl, 2011).

The EPs-7630 experiments with regard to cytokine expression gene (iNOS, IFN- α , IFN- γ , TNF- α , IL-1, IL-10, IL12, IL-18) have shown the immunomodulatory effect of *Pelargonium* in response to infected macrophages (Kolodziej & Kiderlen, 2007). Similar results were obtained in experimental animals considering the anti-infective and immunomodulatory effect of *Echinacea* and *Pelargonium*. In one study, mice were administered once a day (for 7 days) with *Echinacea* extracts and immunized with sheep red blood cells (sRBCs). Antibody response to sRBC was significantly increased by three *Echinacea* species. They caused a significant increase in interferon- α production, but inhibited the release of TNF- γ and IL-1- β . The inhibition of TNF- α was also seen in this study except in one study group. *E. angustifolia* and *E. pallida* administered mice demonstrated significantly higher production of IL-4 and IL-10. These results indicated that *Echinacea* modulates both innate and adaptive immune responses (Zhai et al., 2007a). In a murine model, *E. purpurea* activated macrophages to stimulate IFN- γ production (Mishima et al., 2004).

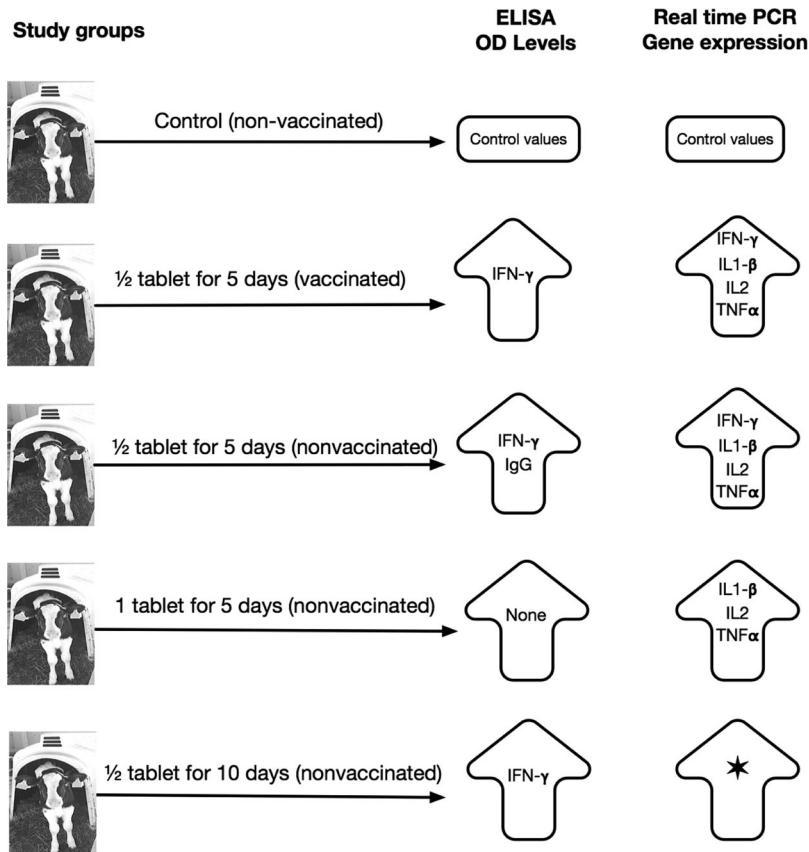


Figure 5. Summary of the statistically significant increase in ELISA and real-time PCR gene expression results of study animals. *Indicates the increase in all groups including controls (accurate level of gene expression cannot be calculated in this group).

In a study performed in rats by using *Echinacea* and *Ginseng*, an increase in expression of IL-10 and TNF- α genes were found (Ulusik & Keskin, 2012). In mice, IMMUNAL was found to be effective in enhancing antibody production to sRBCs but no such effect was seen when *ECHINACEA* FORTE drops used (Bałan, Sokolnicka, Skopińska-Różewska, & Skopiński, 2016). In one study, all the *Pelargonium* extracts (EC(50), gallic acid (EC(50) and its methyl ester (EC(50) significantly decreased the intracellular survival of *L. donovani* amastigotes within murine macrophages by triggering macrophage activation (Kayser et al., 2001). As a murine model of tuberculosis, *Pelargonium sidoides* stimulated macrophage killing, but not uptake of *M. tuberculosis* and *M. fortuitum* equally well (Kim, Griffiths, & Taylor, 2009).

Animal experiments have shown that a combination of *Echinacea* extracts demonstrated greater effects than extracts of a single plant (Bodinet et al., 2002). Therefore, two plants, *Echinacea* and *Pelargonium* were used in this study. However, using a mixture of plants makes it difficult to distinguish the biological significance of each plant or identify redundancy in effects (Bodinet et al., 2002). Results of this study, combination of both plants were tested, indicated that this combination resulted in increase

in γ -interferon levels in three groups of treated animals, and total IgG level in a group of treated animals which received CEVASTIM $\frac{1}{2}$ tablet for 5 days as well as upregulation of targeted cytokine gene expressions except IL-4.

Interesting part of this study was that $\frac{1}{2}$ tablet of the extract used for 10 days induced upregulation of cytokine gene expression but upregulation was also seen in the controls and therefore cannot be concluded as positively correlated with fold changes. This might be associated with several factors such as duration and dose of the extract used. This effect is most likely associated with duration since an increase was observed in all targeted cytokine gene expression, total IgG and γ -interferon levels when CEVASTIM used for $\frac{1}{2}$ tablet for 5 days. All of these effects were not seen in other treated animal groups while some groups were partly responded.

Conclusion

Using $\frac{1}{2}$ tablet CEVASTIM in calves for 5 days has a potential to induce natural immune response as a result to modulate innate and adoptive immunity by increasing IgG and γ -interferon levels and upregulation of important cytokine (γ -interferon, IL-1- β , IL-2 and TNF- α) gene expression in calves. Summary of the statistically significant increase in ELISA and real-time PCR gene expression results are illustrated in [Figure 5](#).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by CEVA Animal Health and the University of Istanbul, Veterinary Faculty Istanbul.

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