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Immunological Influences of the *Calendula officinalis* Tea Treatment in Dairy Cows

Aurel VASIU[1*], Carmen Dana ŞANDRU[1,2], Emoke PALL[1,2]
Janos NANASI[1], Florina MARIAN[1], Diana OLAH[1], Marina SPÎNU[1,2]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine, Division of Infectious Diseases, University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology, 557085, Cristian-Sibiu, Romania
*Corresponding author : aurel_vasiu@yahoo.com

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Abstract

The influence of environmental factors and growth technologies has a substantial impact on health and immunization of animals following antigenic stimulation, so the way vaccine protection is provided cannot be evaluated outside the habitat framework. Medicinal plants have often been used to augment the immune response in humans or animals. In the case of bovine, due to technologies where access to grazing is increasingly available, the animals can benefit directly from the adjuvant influences of medicinal plants in the spontaneous flora.

This research aimed at quantifying the effects of orally administered *Calendula officinalis* aqueous extract (tea) on the innate and adaptive cell-mediated immunity under vaccination conditions, trying to define its immune modulating effect in young bovine. For that, an experiment was performed by administering Calendula officinalis tea perorally to a batch of bovine vaccinated with a complex vaccine for a week post vaccination. On days 0, 7 and 14, *in vitro* leukocytes blast transformation, and the stress level expressed by the N/L ratio were determined. Vaccination is a stressful event for animals; however, the animals subjected to tea treatment had a higher initial N/L index, which decreased considerably, supporting the decrease in the level of stress by administering marigold tea. The in vitro capacity of the mononuclear cells sampled from the marigold tea treated group was higher towards the standard mitogens PHA M and LPS ($p<0.01$-$p<0.05$).

Both Calendula officinalis and *Echinacea purpurea* alcoholic extract acted stimulating, both when compared to the untreated control and in vitro alcohol treated cultures (solvent control). The experimental results indicated that the Calendula officinalis tea has detectable immunological activity in cattle after oral administration for one week. It's administration to dairy cows facilitated the reduction of post-vaccination stress, thus proving its usefulness in amplifying the immune response under antigenic stimulation conditions. The active principles in *Calendula officinalis* tea caused a continuous active increase in cell-mediated immunity, as observed by the blast transformation test, suggesting further studies for tailored treatment.

Key Words: systemic immunity, tea, *Calendula officinalis*, dairy cows, vaccination

1. Introduction

Cattle breeding is a major branch of world's agriculture, due to the volume, diversity and economic value of the products obtained. Cattle provide 95% of the total amount of milk consumed globally, 30-35% of the meat and 90% of the total hides processed in the world's tanning industry (Maciuc et al. 2015). The milk and meat of bovine contains proteins with a high level of availability, digestibility and energy value compared to vegetable proteins, thus contributing to a balanced human diet. The cost/benefit ratio in the case of beef is very convenient, these animals exploiting a wide range of bulk fodder: pastures, coarse feed and others (Galyean and Hubbert, 2014; Richeson et al., 2019; Rusu, 2005, 1981).

Constant exposure of the bovine to physical, chemical and microbial aggressors, sometimes as part of raising technologies, could lead to biased productions and could impact not only on their health and welfare but on the entire production chain, including consumers and the economy as a whole (Kappes et al., 2023, Pirestani et al., 2023).

The immune system safeguards at all times the health of the animals against microbes, therefore it represents a very sensitive indicator of their ability to cope with the habitat, for instance, intensification of productions in confinement conditions found on intensive farms (Hayek, 2022). Combined intensification of farming and emergence of infectious diseases undermine the sustainability of dairy farming, vaccination providing some benefits (Barrington and Parish, 2001). Hence, vaccination as a preventive procedure could be efficient only when the immune system is capable of an adequate response to the vaccine antigen. Nevertheless, there is deficient information and understanding of bovine immune system, which hinders the setting up of an established set of adequate immunological tests to support optimal bovine health under low input farming circumstances (Vlasova and Saif, 2021).

Medicinal plants have been used to augment the immune response in humans or animals. As such, marigold (*Calendula officinalis* L.), a medicinal plant with multiple uses, belongs to order Asterales, Family: Asteraceae and Genus: Calendula L. These are annual plants, with a height of 20-40 cm, very branched. The stem and leaves are sticky and the flowers are ligulate, hermaphrodite. The fruits are sickle-shaped or arched boat-shaped while the fruit are achenes that form rings, with small spikes on the surface (Mihăescu, 2008; Preda 1989). It is widespread, being present in warm temperate regions of the world. From the 25 species of the genus Calendula, due to its active principles, the species Calendula officinalis is mostly used. The therapeutic properties of marigolds are determined by biologically active substances: isoprenoid compounds (volatile oils, saponins, triterpenic alcohols, carotenoids); phenolic compounds (flavonoids, phenolic acids); fatty acids (calendic acid: 60-70%)(Azhar et al., 2023, Abedi et al., 2020, Cushnie and Lamb, 2005); polysaccharides (pectins, hemicelluloses); compounds with nitrogen (alatoniana, proteins and amino acids); other substances (vitamin C, saturated hydrocarbons, minerals – K, Ca, Mg, Mn, Cu, Zn, etc.)(Shahane et al., 2023). Calendic acid, the main constituent (60-70%) of C. officinalis is an omega-6 unsaturated fatty acid (pubchem.ncbi.nlm.nih.gov /compound/Calendic-acid).

The plant shows hepato-protective and antioxidant effect, but is also effective against inflammatory processes, cancer, parasites (helminths), regulates the blood sugar (antidiabetic) and heals wounds (Moradkhani et al., 2015, Shahane et al., 2023). Triterpenes in Calendula extracts have only a minor role in wound healing, but carotenes and xanthophyll derivatives produce a better effect (Nicolaus et al., 2017). *Calendula officinalis* tincture promotes

wound healing by stimulating fibroblast proliferation and migration (Dinda et al., 2015).

The European Medicines Agency (EMA) has approved lipophilic and aqueous alcoholic extracts of Calendula officinalis as traditional medicines for the treatment of minor skin inflammation and as an aid in wound healing. In semi-intensive raising systems, the increasing access of the animals to pasture could enhance the adjuvant influences of medicinal plants from the spontaneous flora. For this reason, studies on the positive effects of medicinal plants on farmed animals, such as bovine, are expanding.

This research aimed at quantifying the effects of orally administered *Calendula officinalis* aqueous extract (tea) on the innate and adaptive cell-mediated immunity under vaccination conditions, trying to define its immune modulating effect in adult bovine.

2. Material and Methods

2.1. Material

2.1.1. Biological materials

Animals: The experiment was carried out on a dairy cow farm, accommodating 100 animals. The cows were raised in a semi-intensive system, in the winter in a free-stall shelter, and between May and November in a pastoral system. During the winter, the animals benefited of a ration consisting of alfalfa hay, grass hay, corn silage, corn meal, wheat bran while during grazing, the ration was supplemented with corn meal and wheat bran. Preventive procedures on the farm included vaccination against the bovine respiratory complex (bovine respiratory syncytial virus, rhinotracheitis, parainfluenza) and viral diarrhea as well as vaccination of pregnant cows during the 6-8 months of gestation with a vaccine against neonatal calf diarrhea (*E. coli,* F5, rotavirus, coronavirus). The animals were dewormed 2-3 times a year, before the grazing period using 1%Ivermectin and Triclabendazole, and upon entering the stable with Ivomec Plus. Protection against flies and Tabanidae during grazing with Cifluthrin was implemented.

Protocol. This experiment was carried out on 20 dairy cows from the Romanian Spotted breed, aged 4 to 6 years. The cows were divided in two equal batches, the first being treated with Calendula tea, and the second batch serving as untreated control. Blood samples were collected from both groups on days 0, 7 and day 14 of the experiment. Blood was collected on heparin (50 IU/ml) and processed within 4 hours after sampling.

On day 0 of the experiment, the both groups were vaccinated with a combined vaccine containing multiple antigens: bovine respiratory syncytial virus, bovine rhinotracheitis virus, parainfluenza virus. Batch 1 received the Calendula tea for 7 days in a dose of 1l/individual, a single administration per day. For preparing the tea, 200 g of dried marigold (Flores Calendulae, Farmanat Poieni, Romania) was added to 15 l of boiled water and the mixture was left to infuse for 15 minutes, after which it was filtered, cooled and ready to use (Vasiu et al., unpublished data).

2.2. Method

Total leukocyte counts (Blumenreich, 1990): Immediately after each sampling (0, 7 and 14 days), blood smears were made and stained with Dia-Quick Panoptic staining kit (Reagent, Budapest). Stained blood smears were examined under a microscope (100x magnification) and the white blood cells were counted, then the N/L ratio was calculated for each sample. The N/L ratio was interpreted as a stress indicator, while its value increased towards value 1 of the ratio. The lower the value of N/L ratio is, the less stressed the animals are. Leukocyte blast transformation test (Khokhlova et al., 2004). To cultivate the blood cells, RPMI 1640 cell-culture medium supplemented with 10% fetal calf serum (SFV), 1000 IU penicillin and 1000 µg

streptomycin/ml, pH 7.2-7.4 was used. To test the in vitro ability of the cells to grow, 1.92 ml RPMI were mixed with 0.48 ml of blood and distributed in aliquots of 200 µl/well in 96 well plates. Additionally to the untreated control cultures, experimental variants treated with mitogens (PHA M and LPS, 1 µl/well), alcohol (solvent control, 1.5 µl/well) as well as alcoholic extracts of Calendula officinalis and Echinacea purpurea (Lochman, 2022)(1.5 µl/well) were performed. All samples were done in duplicate. The plates were then incubated in a 5% CO2 atmosphere for 72 h.

At the end of the incubation period, the glucose residue was quantified from the culture supernatant by the orto-toluidine colorimetric method. For that, 12.5 µl of supernatant were added to 0.5 ml o-toluidine reagent. The mixture was kept for 8 minutes in boiling water, then it was suddenly cooled and the color was red by a spectrophotometer (SUMAL PE2, Karl Zeiss, Jena) against a glucose standard of 100 mg%, processed under the same conditions. Depending on the glucose residue values, the stimulation indices were calculated according to the formula: TI%=[(CMG–RG)/CMG]´100, where TI represented the blast transformation index, CMG the glucose concentration in the initial RPMI and RG the glucose residue in the sample subsequent to incubation.

2.3. Statistical analyses

Mean values, standard deviations and the statistical significance of the differences (t-Student test) between all the results obtained for the two batches and for different experimental variants were calculated by use of Microsoft Excel program, version 2010.

3. Results and Discussion

In Romania, cattle breeding represented an economic priority (Morar, 2007). The milk production continuously increased during the past three years, with a plus of 4.6% in 2024 when compared to 2023, based on local production rather than imports, which decreased with more than 50% during the same period of time (National Institute of Statistics, Romania, INSSE). Thus, considering that milk and dairy products supply a significant percentage of the local population's food, on-farm health programs are very important in ensuring not only dairy cows' health but also consumers' health. Prevention of diseases caused by microbes in farmed animals, including dairy cows, is achieved by vaccination, antibiotic therapy and chemoprophylaxis.

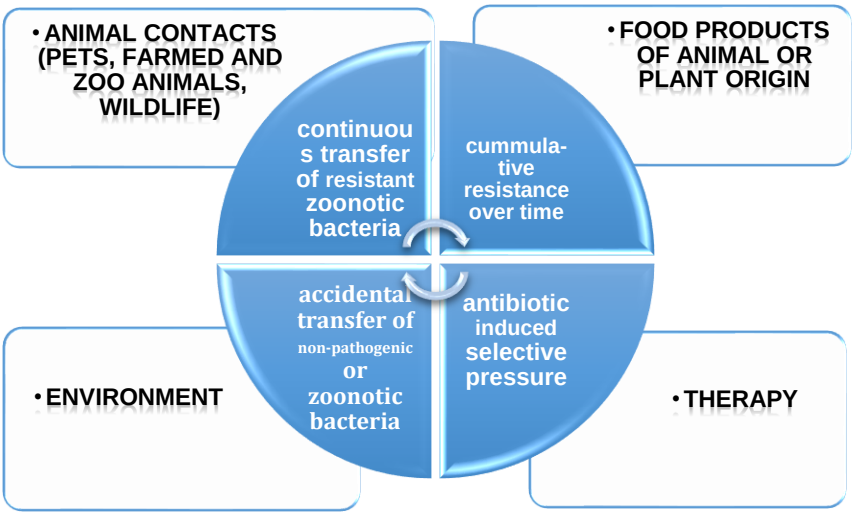


Fig. 1. Main sources for human exposure to antibiotic resistant bacteria (Spinu et al., 2018)

The World Health Organization emphasized the need to use alternative methods in the current frame of the excessive and irrational use of antibacterial and anti-parasitic products and rapid progression of antimicrobial resistance. The widespread indiscriminate (no antibiotic resistance test) and injudicious (excessive doses) use of antibiotics in the treatment of diseases in humans and animals have gradually led to the establishment of the antibiotic resistance phenomenon.

In Fig.1 the main sources for the appearance of antibiotic resistance in humans were presented, underlining the importance of the intervention of the environment, the animal sector and food of animal origin, in addition to the actual involvement of humans. The strong selective pressure exerted by antibiotics lead to the increase of the MAR index and the induction of the multi-antibiotic resistance, a frame where common bacterial diseases could not be controlled by antimicrobial therapy (Cernea et al., 2015, Motalebipour and Pirestani, 2022). The usefulness of medicinal plants in the therapy of various ailments over time has led to the validation of natural products with multiple effects (Daglia, 2012).

Calendula officinalis was studied along time for its multiple beneficial effects. In a comparative study of wild and cultivated marigold, Cetkovic et al. (2004) indicated that methanolic and aqueous extracts of the plants in concentration from 0.10mg/ml to 0.90mg /ml effect scavenged all types of radicals used (2,2-diphenyl-1-picrylhydrazyl-DPPH), hydroxide radical, and lipid peroxide radical - ESR) depending on their concentration. Nevertheless, the cultivated marigold had a higher antioxidant effect than the the wild plant, while the methanol solvent interfered with the activity, the aqueous extracts producing a higher antioxidant effect. 0.75mg/ml of the aqueous extract of C. officinalis completely eliminated the hydroxyl radical and scavenged 92% DPPH and 95% peroxide radical through lipid peroxidation. The authors considered that this high antioxidant activity was due to the total phenolic (14.49-57.47 mg/g) and flavonoids (5.26-18.62 mg/g) content. They also compared the methanolic and aqueous extracts of cultivated marigold showed antioxidant properties comparable to those of butylated hydroxyanisole (BHA) a synthetic antioxidant (Cetkovic et al., 2004). Similarly, the orraly administerd *Calendula officinalis* aqueous extract had an antioxidant and anti-inflammatory effect in experimental periodontitis in rats (Lima et al., 2017).

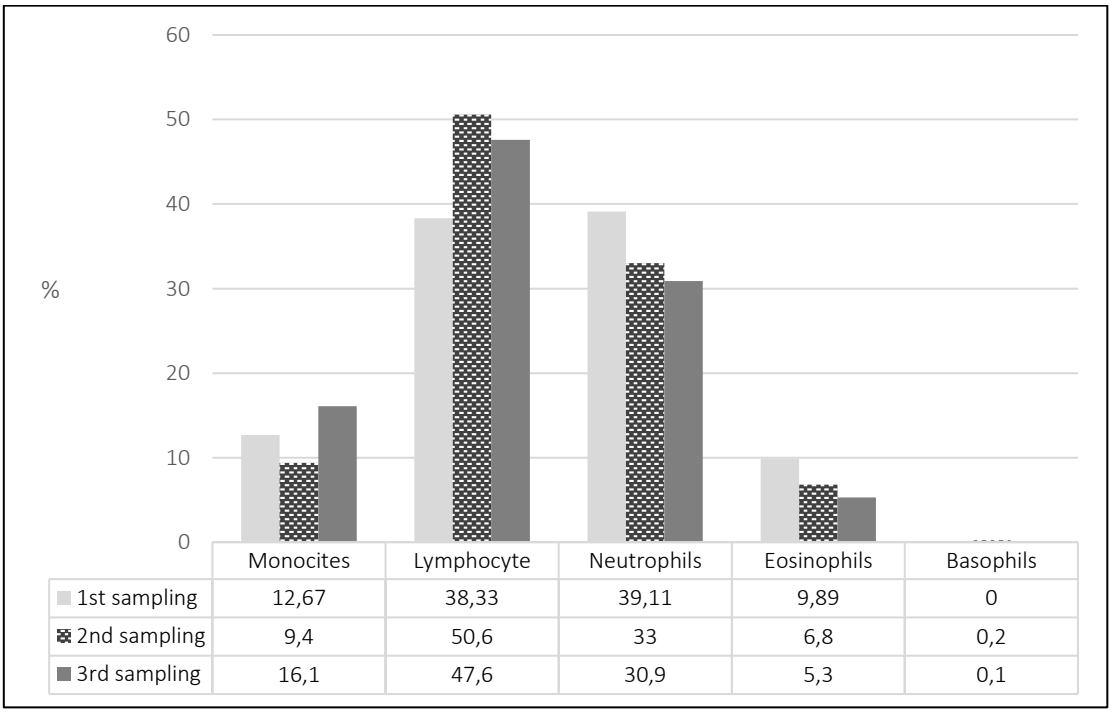
Further, a dose-dependent wound healing effect of the alcoholic extract of Calendula officinalis administered orally has been demonstrated (Preethi et al., 2009). Another potent therapeutic component derived from Calendula officinalis is the essential oil based on its properties to prevent and reduce the oxidative stress (Stef et al., 2009, Lohani et al., 2019). These researches stands for the very important antioxidant activity of the marigold extracts, as a therapeutic means in animals.

Although the aim of this study was not to evaluate the antioxidant capacity of the marigold tea administered to the cows, it was presumed that the immunological changes were, to some extent, reliant on the antioxidant activity of the aqueous plant extract. Considering that:

a) Resistance to antibiotics is constantly increasing and antibiotics end up in food of animal origin, promoting the increase in the antibiotic resistance of the bacteria in humans, and

b) Treatments with plant extracts are well tolerated, cheap and do not induce resistance, Calendula officinalis tea was administered to dairy cows, with subsequent monitoring of its immunological potential in cattle vaccinated with a multivalent vaccine.

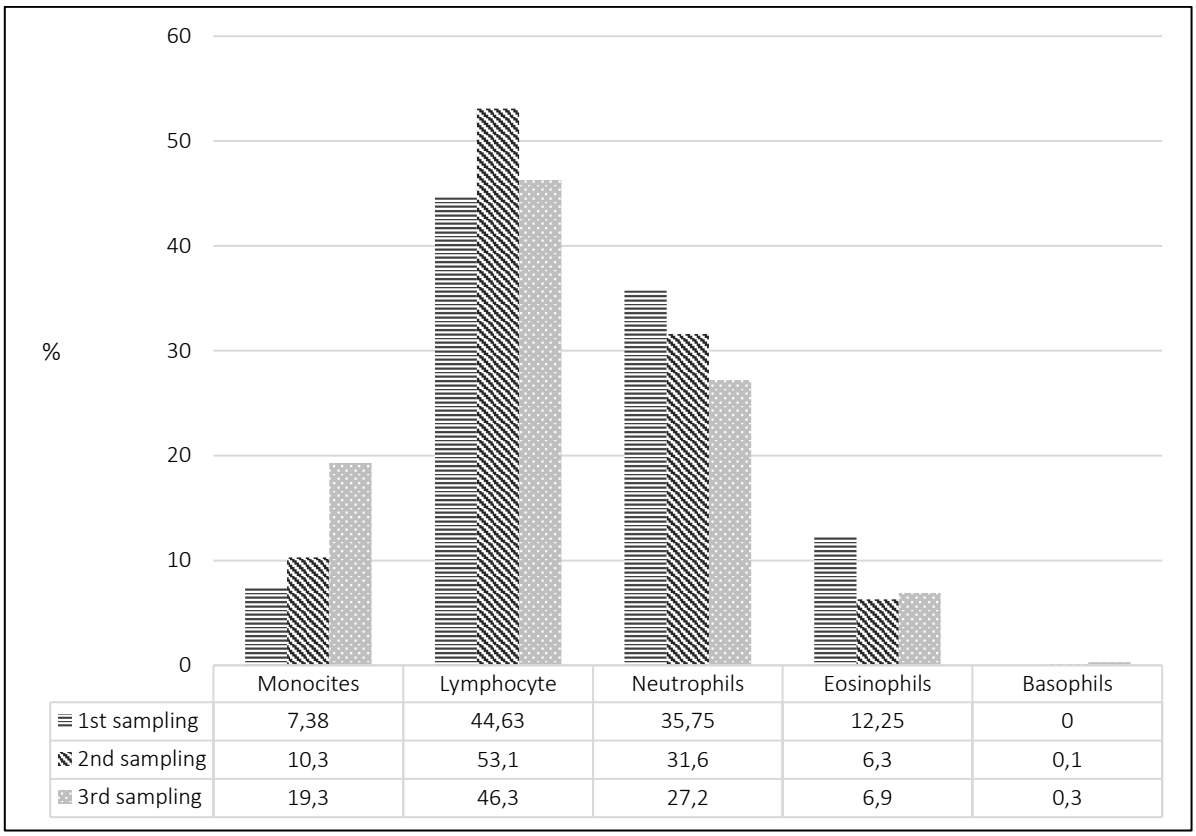
Davis et al. (2008) synthesized the usefulness of leukocytes counts and calculation of the N/L ratio and demonstrated that in stressful conditions, there are changes in the leukocyte population, with the number of lymphocytes decreasing and the number of neutrophils increasing. It seemed that this indicator was more trustworthy than the level of circulating corticosteroids induced by any kind of stress. Figures 2 and 3 represented the changes in leukocyte sub-populations, during the experiment in the Calendula officinalis tea treated animals and the untreated control group. When comparing the two groups, it becomes obvious that the dynamic's pattern differed in monocytes, where in the first group it was a decrease-increase pattern in the tea treated group while in the control group there was a steady increase, with final values non-significantly higher than in the experimental animals. In the lymphocyte subpopulation, the pattern was similar, going through an increase and subsequent decrease in both groups, this time with a higher value in the tea treated group. Nevertheless, the increase in lymphocytes subsequent to the administration of the tea was higher (32.01%) than in the untreated group (18.97%, $p<0.05$) at the second sampling and increasing (24.18% versus 3.74%, $p<0.01$) by the end of the experiment.



	Monocites	Lymphocyte	Neutrophils	Eosinophils	Basophils
1st sampling	12,67	38,33	39,11	9,89	0
2nd sampling	9,4	50,6	33	6,8	0,2
3rd sampling	16,1	47,6	30,9	5,3	0,1

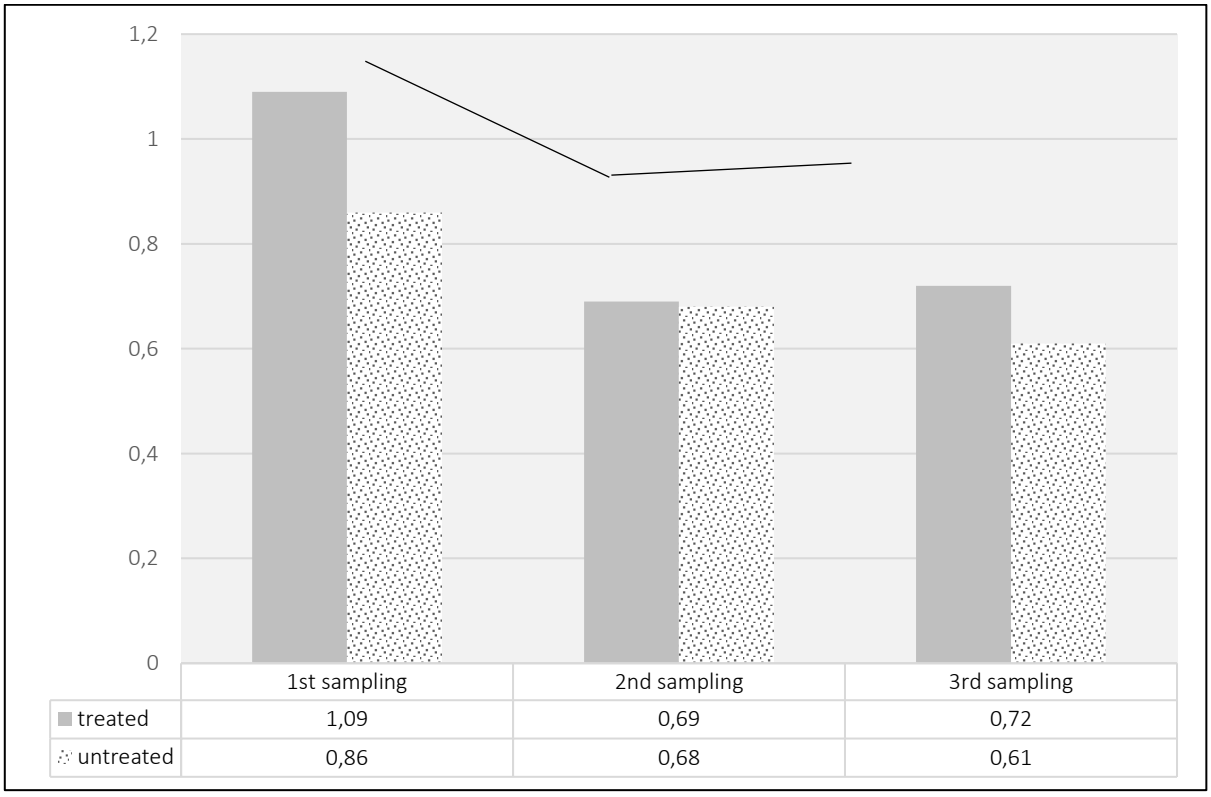
Fig. 2. The dynamics of leukocyte populations in *Calendula officinalis* tea treated animals (average values)

The neutrophile (15.62% -2nd and further 6.36% - 3rd sampling) and eosinophile (31.24% and 22.06%, 2nd and 3rd samplings, respectively) percentages constantly decreased in the tea treated group (Fig.2). There was a similar response of neutrophils (11.61% and 13.92%, 2nd and 3rd samplings, respectively) but not of the eosinophils (-48.70% and +9.52%, 2nd and 3rd samplings, respectively) in the untreated group (Fig. 3).



	Monocites	Lymphocyte	Neutrophils	Eosinophils	Basophils
1st sampling	7,38	44,63	35,75	12,25	0
2nd sampling	10,3	53,1	31,6	6,3	0,1
3rd sampling	19,3	46,3	27,2	6,9	0,3

Fig. 3. The dynamics of leukocyte populations in untreated control animals (average values)

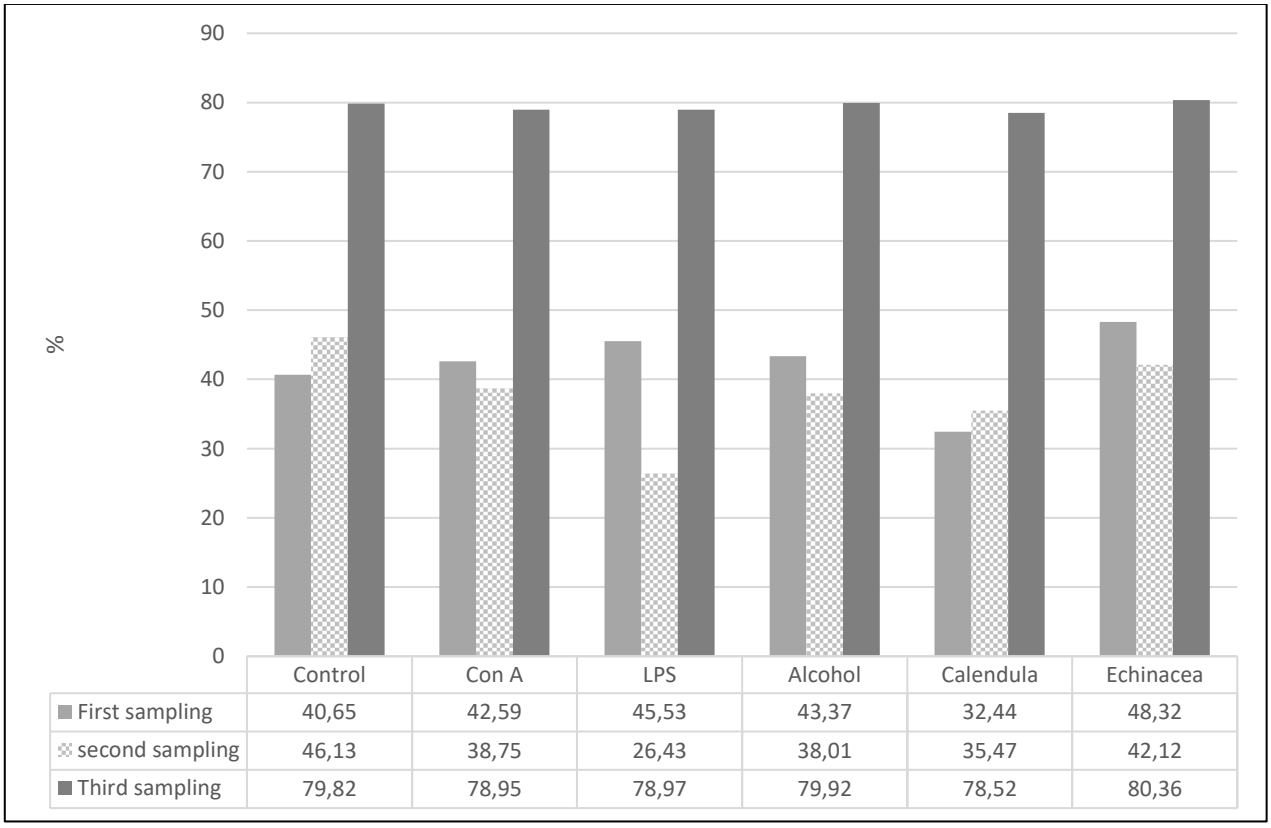


	1st sampling	2nd sampling	3rd sampling
treated	1,09	0,69	0,72
untreated	0,86	0,68	0,61

Fig. 4. The dynamics of N/L ratios in treated animals versus untreated controls

The average neutrophil/lymphocyte ratio (Fig. 4) was higher in the group treated with marigold tea compared to the untreated group at the first sampling (Fig 2). Nevertheless, it evened out until the second sampling, with a non-significant increase towards the third sampling. These results could indicate the anti-stress effect of the marigold tea in the treated group. A more pronounced decrease (37% versus 20.9%) in the stress index could be observed in the group treated with marigold tea, compared to the untreated group, between the first and second sampling. Vaccination is a stressful event for animals, however, the animals subjected to tea treatment had a higher initial N/L index, which decreased considerably, supporting the decrease in the level of stress by administering marigold tea (Arthington et al., 2008, Leach et al., 2013).

The stress level was slightly increased in the group treated with tea, probably due to the administration procedure prolonged for one week, without the differences between the groups being statistically significant (at least $p<0.05$)(Davis et al., 2008, Dudek et al., 2014). In the blast transformation test (Fig. 5-6), there was a substantially increased ($p<0.05$-0.001) TI at the end of the experiment in the tea treated group, versus the initial values and also versus the untreated group, as indicated by the control cultures (96.36 versus 60.06%, $p<0.01$, in the tea treated versus untreated animals, as in Table 1).



	Control	Con A	LPS	Alcohol	Calendula	Echinacea
First sampling	40,65	42,59	45,53	43,37	32,44	48,32
second sampling	46,13	38,75	26,43	38,01	35,47	42,12
Third sampling	79,82	78,95	78,97	79,92	78,52	80,36

Fig. 5. Dynamics of TI indices recorded in the treated experimental animals (x±s).

Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

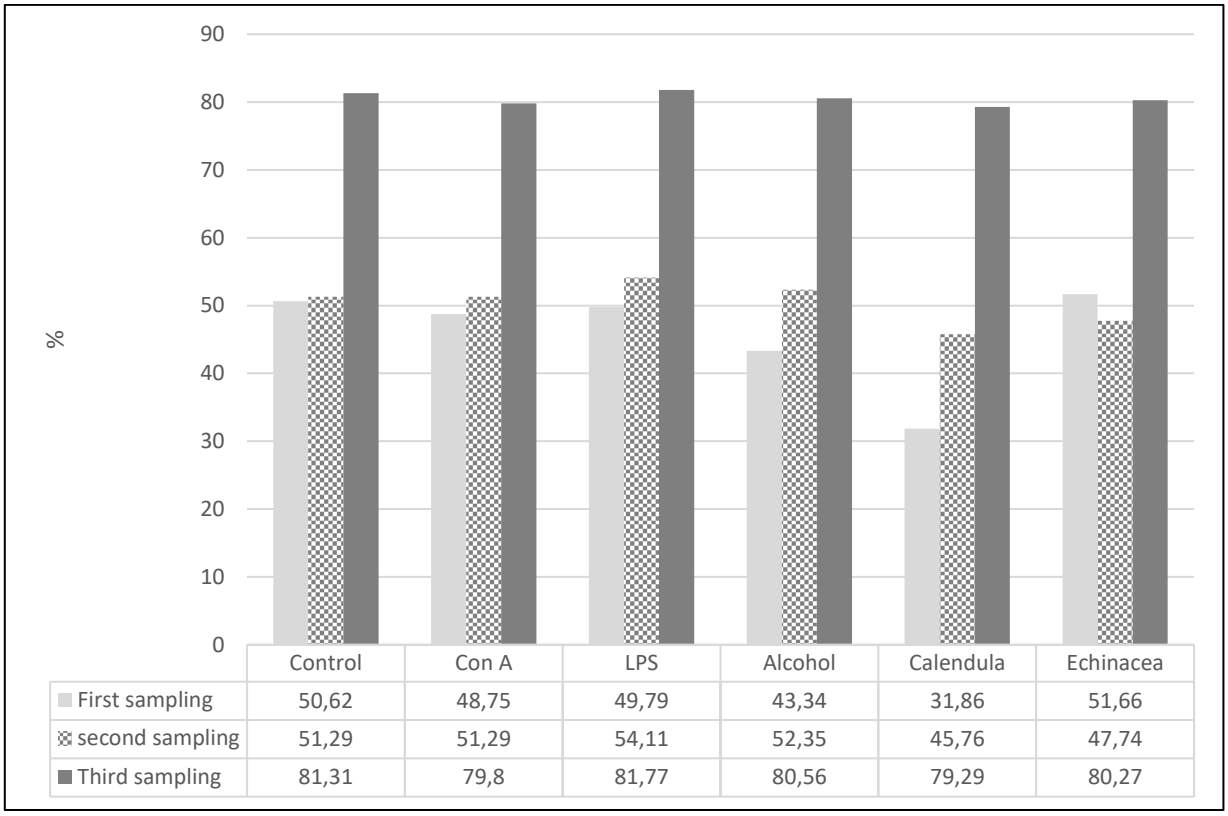


Fig. 6. Dynamics of TI indices recorded in the treated experimental animals (x±s). Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

Table 1. The increase (%) of the TI during the experiment in the two groups

Group	Control	ConA	LPS	Alcohol	*Calendula*	*Echinacea*
Treated	96.36	85.37	75.45	84.27	142.05	66.30
Untreated	60.06	63.69	64.42	85.88	148.87	55.38

Legend: TI%=transformation indices, Control= untreated *in vitro* variant; ConA, LPS, *Calendula, Echinacea* indicated the *in vitro* applied treatments with concanavalin A, lipopolysaccharide from *E. coli*, and *Calendula* and *Echinacea* alcoholic extracts, respectively

This data supported the positive impact of the relatively simple, at hand and cheap preventive treatment formula, enhancing the immune reaction before the vaccination. The quantification of the post vaccination titers, which were not quantified due to the complex composition of the vaccine chosen by the farmer – would add to the value of the obtained results.

From Fig. 5-6 and Table 1 it is obvious that the in vitro capacity of the mononuclear cells sampled from the marigold tea treated group was higher towards the standard mitogens PHA M and LPS ($p<0.01$-$p<0.05$). Both Calendula officinalis and Echinacea purpurea alcoholic extract acted stimulating, both when compared to the untreated control and in vitro alcohol treated cultures (solvent control). Although the differences in the in vitro responses to Calendula extract were similar, the marigold tea treatment enhanced the in vitro response to the Echinacea extract, proving once more its stimulating capacity.

Conclusions

1. The experimental results indicated that Calendula officinalis tea has detectable immunological activity in cattle after oral administration for one week.

2. Calendula tea therapy facilitated the reduction of post-vaccination stress, thus proving its usefulness in amplifying the immune response under antigenic stimulation conditions.

3. The active principles in Calendula officinalis tea caused a continuous active increase in cell-mediated immunity, as observed by the blast transformation test, suggesting further studies for tailored treatment.

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Author Contribution

There was an equal contribution of all authors declare to the study design and experimental work, interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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