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In-vivo evaluation of an innovative feed additive formulation of *Pinus Brutia ten*. resin containing turpentine and colophony and the effects on milk production performance and somatic cell counts of Holstein dairy cows

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ABSTRACT

The aim of this study was to investigate a feed additive which is expected to be preventative, consist of new active ingredients for mastitis disease which is frequently seen in dairy cattle. For these purposes colophony which has antibacterial and turpentine which has antibacterial, antifungal effects have a potential that is researched in vitro previously. These herbal ingredients were suggested to use for prevention of mastitis in dairy cattle. Powdered herbal material have colophony and turpentine %96.5± 0.3 and % 3.5± 0.3, respectively. Animal experiment was studied with two group (experimental, control) which had 15 dairy cattle each and at the end of the study milk parameters, somatic cell count (SCC), average milk productions discussed. Although it was not statistically significant the experimental group had higher milk amount, milk fat and lower SCC than control group within two weeks.

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Introduction

Mastitis is the most common disease in dairy cattle all over the world, which significantly reduces the profitability of dairy farms (Bradley, 2002; Ruegg, 2017). Mastitis occurs when pathogenic microorganisms enter the nipple and cause inflammation in the breast tissue. It is quite easy to diagnose of acute clinical mastitis. Mastitis can be if the structure of the milk has changed and here is an abnormal appearance (watery appearance, flakes, or clots) in the milk (Adkins and Middleton, 2018). Although clinical mastitis is easy to diagnose in this way, subclinical mastitis is a condition that should be followed in the herd, where there is no visible change in the breast structure (redness, burning, pain and abnormal milk) when viewed from the outside, but somatic cell count (SCC) is higher than SCCs in milk from healthy animals' subclinical mastitis also decreases milk yield (Sharma et al., 2011).

Treating this condition creates a high cost per administration when conventional drug forms are used. In addition, excreted of antibiotics with milk prevents the use of milk during the treatment. Since antibiotics are the most preferred method in the treatment of mastitis, it is great importance for human health to prevent mastitis in order to prevent the development of resistance. Subclinical mastitis is a

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condition that there were no acute signs of clinical mastitis but SCC of milk sampled from breast are higher than a sampled milk from healthy breast. Decrease of milk production is most important economic effect of subclinical mastitis. As a result, milk industry always keeps searching for preventative solutions for mastitis. And various methods are used to detect subclinical and clinical mastitis. SCC is the most widely used mastitis detection method, and leukocyte concentration in one milliliter of milk (Moreira et al., 2019). It has been stated that SCC over 50.000 (Seegers et al., 2003), 100.000 (Koç, 2018), depending on the sources, is the diagnostic border between the infected breast and the healthy breast. Studies have shown that increased SCC cause decreases in the range of 1.4-2.7 liters of daily milk production (Dohoo and Meek, 1982).

Considering the above-mentioned mastitis is an important cattle disease and the needs to reduce its incidence, a mixture of colophony and turpentine was selected as a candidate to prepare an oral dosage formulation in order to prevent mastitis. The biggest reason why these active ingredients are preferred is that the first patent application in this field in the world was made in Turkey and remarkable results were obtained from field trials in cattle and many different animals subject to the patent application, apart from the properties examined in cattle.

In today's studies, in addition to antibacterial, antifungal (Savluchinske et al., 1999), antiviral (Savluchinske et al., 1999), anticancer (Tanaka et al., 2008), antiparasitic (Mercier et al., 2009) properties of colophony and turpentine separately or together, it can also prevent pathogens in the intestinal flora with oral use. It is understood that it supports feed efficiency and makes significant contributions to the veterinary field (Kettunen et al., 2015). In addition, it has been observed that abietic acid in colophony and turpentine containing a-pinene have a bactericidal effect against methane-producing bacteria that cause energy loss in cattle (Sierra-Alvarez and Lettinga, 1990). Methanogens uses feed in stomach of cattle to produce methane.

The aim of this study is to observe the effects of the formulation containing colophony and turpentine on milk production, milk protein and fat contents and somatic cell counts.

Materials

Pinus Brutia Ten. Resin and Bentonite gifted from Uğur Yem Katkı Maddeleri Ltd, Türkiye, Calcium Carbonate gifted from Ortaş Ltd, Türkiye, HPMC 10.000 Da, Gum Colophony purchased from Sigma Aldrich, Germany, and carboxy methyl cellulose (CMC 10.000) Da purchased from Zibo Hailan Chemical Co., Ltd, China.
Sonkaya bag closer device (smpy 402 40 CM) purchased from Sonkaya Machinery and Automation Technologies, Turkey.

Methods

The resin used in this study was obtained from the *Pinus Brutia Ten*. plant Gördes, Manisa, Turkey. Its components were used to make an oral reconstituted suspension formulation for dairy cows.
All prepared reconstituted suspension formulations were shown at Table 1.

While preparing the formulations, firstly the powders were weighed and mixed in the mixer according to the geometric dilution method. For easier wetting, CMC cp 10.000 with better water dispersion was preferred as wetting agent. The wetting agent was added to the mixer after mixing the powders. It was mixed until a homogeneous view was obtained, then the mixture transferred to a bottle and mixed with increments of dispersion medium by shaking.

Table 1. Prepared reconstituted suspension formulations

	Formulation code										
Contents (g)	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**	**F16**	**F17**
Colophony/Turpentine Mix.	15	15	15	15	**15**	15	15	15	15	15	15
CMC 10.000 cp				0.2	**0.35**	0.5	0.35	0.35	0.35	0.35	0.35
$CaCO_3$	4.5	6	7.5	6	**6**	6			6	6	6
Starch 1500							6				
Avicel Ph 102								6			
Sodium citrate									0.1	0.3	0.5
Methyl cellulose 4000 cp	0.35	0.35	0.35								
Glycerine											
Distilled water (mL)	50	50	50	50	50	50	50	50	50	50	50

CMC = carboxy methyl cellulose, cp = centipoise, Mix = mixture

The final formulation that is used in experiment was chosen with suspension properties F (sedimentation volume), degree of flocculation (b), redispersion time (s), number of turns (times).

The sedimentation volume (Equation 1), F, is the ratio of the final, or ultimate, volume of the sediment, Vu, to the original volume of the suspension, V0, before the settling.

$$F = \frac{V_u}{V_o} \qquad \text{Equation 1}$$

Degree of flocculation (Equation 2), b, is a better parameter for comparing flocculated systems is the degree of flocculation, b, which relates the sedimentation volume of the flocculated suspension, F, to the sedimentation volume of the suspension when deflocculated, F_∞.

$$\beta = \frac{F}{F_\infty} \qquad \text{Equation 2}$$

After the suspensions precipitated, waited until all particles were below the suspension, redispersion time and number of turns were measured. It was resuspended by turning it 180 degres in a 100 mL graduated cylinder to disperse it again. The number of spins and the elapsed time were recorded. 180 degree rotation was done once in 2 seconds. The second measurement was made until the time it looked like there would be no residue at the bottom. 30 cows used in this study were selected among 120 cows and 15 of cows were allocated as control and 15 of cows as experimental group. Animal characteristics that are used to choose these groups are listed in Table 2 and they had statistically same SCC at beginning of study (p>0,01).

Table 2. Animal characteristics of the groups

Animal breed	Holstein
Age (Month)	42-116
SCC	>50.000
Live weight	550-650 kg

SCC = somatic cell count

A reconstitute suspension formulation was chosen to apply the cattles orally. After adding 100 ml of water on the powder form of the combination and shaked well, then the formulation ready for applying orally. Density, Housner ratio, compressibility properties were performed on this powder combination (Jan, 2009).

In order to conduct the in-vivo experiment, the ethics committee approval dated 13/01/2017-17793 was obtained from the Istanbul University Rectorate Animal Experiments Local Ethics Committee.

The experimental period was designed as 30 days. Care was taken to ensure that both of the groups of animals included in the experiment were above 50.000 somatic cells/ml. The SCC of the 25 animals included in the experiment was above 100.000 cells/ml.

To avoid the effects of environmental differences, the animals were fed under the same conditions and on the same ration.

The dose is 2.1 mg/kg for 500 kg live weight dairy cattle used in the experiment. During trial, each milking cow in experimental group take orally 30 g Pinus Brutia Ten. resin which has colophony and turpentine 96.5% ± 0.3, 3.5% ± 0.3 respectively. Resin formulated as an oral reconstitute suspension formulation. Control group and experimental group had same feed ration and ad libitum water.

Somatic cell count fat, protein content in the milk samples taken on the same day of each week and average of weekly milk amount (L) of the experimental group and the control group were compared with the non-parametric Mann Whitney U test in SPSS (IBM Statistics Ver 23).

During the study, 40 ml milk samples were taken from each dairy cows once a week on the same day. During the transportation of milk samples, chemical tablets that stop microbial growth without affecting the somatic cell count (SCC) and milk components were used to prevent the deterioration of the structure of the milk by using “(Microtabs II)” and the milk samples were taken to the “Istanbul University Faculty of Veterinary Medicine Department of Animal Science” Laboratory within the same day in the cold chain. After the milk samples were heated in a water bath at 40°C and somatic cell count, fat, protein, lactose and dry matter values were determined. In the analysis, the "Combi 150" (Bentley), which was created with the integration of the cell counter (Somacount 150) and milk components measuring device (Bentley 150) and works with the "Flow cytometry analysis method" in the "Laboratory of the Department of Animal Science", together. The current device and the analysis methods it used were approved by “ICAR: The Global Standard For Livestock Data”.

Stability tests of final formulation were performed according to the accelerated 6-month test criteria in the International Council for Harmonisation (ICH) Q1a (R2) guideline.

Results

Results of in vitro studies: Properties of powder are listed in Table 3.

Table 3. Powder properties of the formulation (Mean ± SD)

Density ($r_{cluster}$)	0.444 g/ml ± 0.022
Density ($r_{compressed}$)	0.628 g/ml ± 0.043
Housner ratio	1.414 ± 0.028
Compressibility (%)	32.669 ± 3.449

All formulations properties of prepared reconstituted suspensions were shown in Table 4.

Table 4. Data of suspension controls of prepared reconstituted suspension formulations (* (Mean ± SD) The values of the formulations that do not use flocculating agents have not been calculated)

Formulation code	Flocculation volume (F)	Degree of Flocculation (*b*)	Redispertion time (sec)	Number of turns (time)
F7	0.589 ± 0.023	-*	67 ± 2.65	35 ± 1
F8	0.641 ± 0.003	-*	66 ± 3.61	32.6 + 3.06
F9	0.624 ±0.018	-*	77.6 ± 2.52	43.3 ± 1.53
F10	0.548 ± 0.014	-*	57.3 ± 2.52	30.3 ± 1.53
F11	0.853 ± 0.025	-*	48.3 ± 1.53	24.3 ± 2.31
F12	0.989 ± 0.009	-*	139 ± 3.61	70 ± 1
F15	0.974 ± 0.009	1.144 ± 0.036	59.3±0.58	32.3 ± 1.16
F16	0.979 ± 0.009	1.149 ± 0.036	60 ± 4.36	29.6 ± 2.52
F17	0.995 ± 0.009	1.168 ± 0.031	78 ± 2.65	43.3 ± 1.53

Viscosities of the formulations were examined by Brookfield (USA) rotating shaft viscometer. Viscosities of F10, F11, F12 formulations, which is advantageous for application and storage compared to other formulations in terms of redispersion time and settling volume (Table 5). The viscosity increases as the rpm increases, depending on the ratios of the suspension agent in these formulas.

Table 5. Viscosity for F10, F11, F12 formulations (n=3)

Spindle 2/ 30 rpm	F10 Mean ± SD	F11 Mean ± SD	F12 Mean ± SD
1' Cp	117.3 ± 9.7	624.7 ± 4.7	1701.3 ± 66.9
1' Torque	8.9 ± 0.7	46.7 ± 0.1	51 ± 2
5' Cp	84.3 ± 0.6	592 ± 12.5	1641.3 ± 43.3
5' Torque	6.3 ± 0.1	442 ± 0.6	49.2 ± 1.3

F11 formula was preferred as the formulation to be used in oral administration of active substances in animal experiments, due to its redispersion time, settling volume, a high active substance/filler ratio, and also closest F value to "1" (Table 4 and Table 5).

Results of some specifies of F11 Formula was shown in Table 6.

Table 6. Results of some specifies of F11 formula (n=3, Mean ± SD)

Density ($r_{cluster}$)	0.664 g/ml ± 0.004
Density ($r_{compressed}$)	0.843 g/ml ± 0.007
Housner Ratio	1.269 ± 0.002
Compressibility (%)	21.232 ± 0.158
Stack Angle (a)	37.251 ± 3.783
Weight Deviation (g) (packaged)	43.3783 ± 0.1149
Content Uniformity (g) (Amount of insoluble in organic solvent)	12.891 ± 0.079
Particle Size (d values of the semi-logarithmic results) (µm)	d_{10}= 45.236 d_{50}= 136.346 d_{90}=410.96

Results of animal experiments : Number of SCC in the milk, Logritmic SCC, milk fat percentage, milk protein percentage and average milk volume results are depicted in Figures 1-5.

The first week shows the results before the start of the trial and is marked as + in all Figures. Values from 2nd to 8th week of the trial shows the trial results. The results from 8th, 9th and 10th weeks show the results after the trial completed and pointed with "*" on figures.

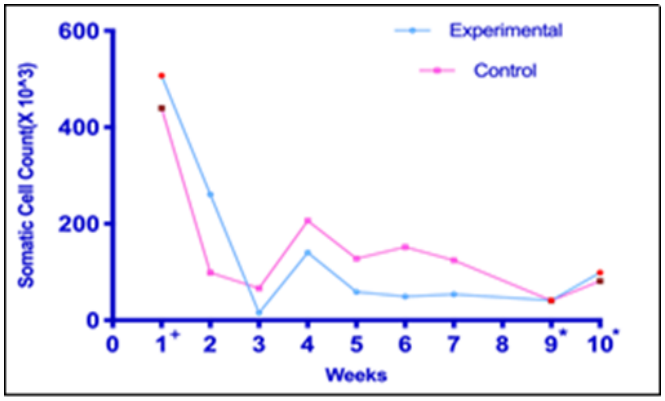

Figure 1. Weekly change in the number of SCC in the experimental and control groups.

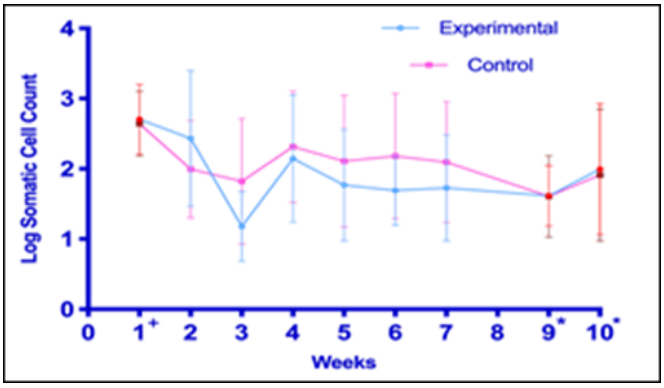

Figure 2. Weekly change in logarithmic SCC in the experimental and control groups.

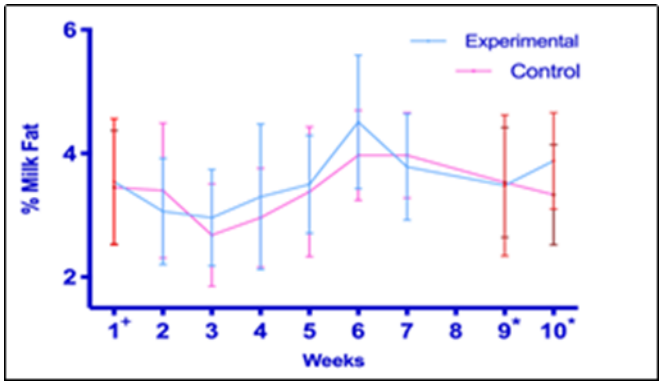

Figure 3. Weekly change in milk fat percentage in the experimental and control groups

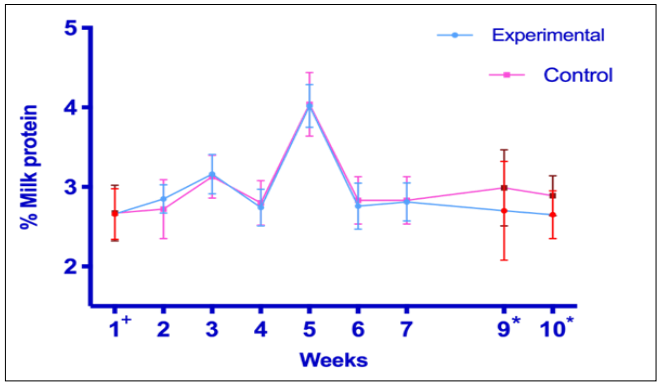

Figure 4. Weekly change in milk protein percentage in the experimental and control groups.

Furthermore, in Figure 1 showing the somatic cells, Figure 5 showing the weekly milking averages, it was seen that SCC decreased more in the experimental group than the control group within 2 weeks from the day the formulation started to given and the milk amount was in favor of the experimental group, and approximately 1 liter increase was observed. In the 7th week when the experiment ended, it was seen that SCC increased again in the experimental group compared to the control group, and the amount of milk decreased compared to the control group.

No statistically significant difference was found in % milk fat (Figure 3) ($p>0.05$). No difference was observed in weekly values between the two groups in milk protein (Figure 4). However, although there is no statistical difference in Figure 3, where milk fat % are shown, it was seen that the amount of milk fat increased in the experimental group in the 2nd week and remained higher than the control group until the 6th week.

In both groups, a decrease in the amount of milk in the summer period was expected, but the decrease in SCC was beyond the expected.

There were no significant difference between experimental and control groups for SCC, Log SCC, milk fat%, milk protein%, weekly average milk amount (L).

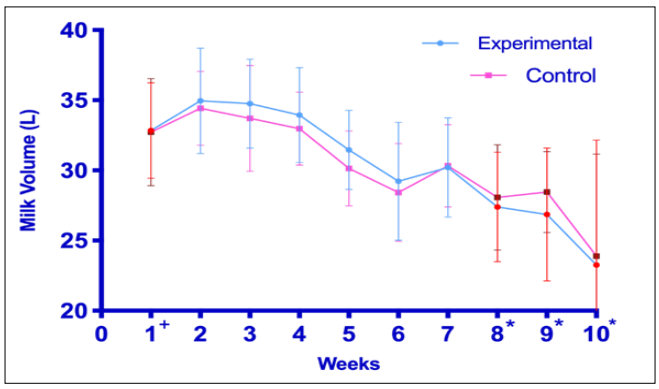

Figure 5. Weekly change in average milk volume in the experimental and control groups.

The selected formulation has successfully passed the ICH accelerated stability tests, and there has been no change in the major components of the herbal content above the criteria set by ICH Q1a(R2).

Discussion

There were no studies of the oral use of rosin (a resin from colophony) and turpentine before with cattle. Antibacterial (Dığrak et al., 1999; Roy, 2018) antifungal (Savluchinske et al., 1999) antiviral (Gigante et al., 2003) anticancer (Tanaka, 2008), antiparasitic (Mercier et al., 2009) properties have been demonstrated in vitro when rosin and turpentine are used alone or together. In addition to the antimicrobial properties of turpentine and rosin, it has been shown to suppress methanogenic bacteria in the rumen (rumen) at a concentration of 43-330 mg/L. Methanogens are bacteria that cause energy loss by producing methane gas in cattle (Sierra-Alvarez and Lettinga, 1990).

In studies of an invention made with a patent obtained in Finland (Patent number: FI124918), it was found to be effective against the mastitis agent S. Aureus in the study performed with the broth dilution method at a concentration of 0.1% (g/L) of resin acids. It was found to be effective against some strains of E. coli at a concentration of 0.5% (g/L). Inhibition was also detected against C. Perferinges, the causative agent of necrotic enteritis, which is a digestive system disease, even at a concentration of 0.01 (g/L). The subject matter of the patent and studies contains 8% resin acid. The effective concentration against S. aureus and E. coli is at 5 g/L (Roy et al., 2018; Kettunen et al., 2015).

When turpentine was used alone, the MIC value against S. aureus, E. coli and C. albicans was found to be 68 mg/ml (Ulukanlı et al., 2014). The volume of rumen, which is the largest compartment of the stomach in cattle, increses as the weight of the cattle increases up to 50-120 liters (Moran, 2005). The resin acids achieved the rate of 96.5% ± 0.3 (w/w) in 30 g

rosin turpentine. The desired MIC concentration, in order to suppress the pathogenic microorganisms and methanogens in the rumen, cannot be achieved by the turpentine at the rate of 3.5 ± 0.3 (w/w).

Toxicity studies of colophony and turpentine were also investigated. In particular, toxicity studies on oral administration are limited. Especially, studies for turpentine have focused on inhalation and skin exposure (Saeidnia, 2014). Although the toxic doses found for the turpentine part are lower than the doses found for rosin, the toxic doses determined for animals in the study are not reached for both substances. While the LD50 value for turpentine is 5760 mg/kg (Saeidnia, 2014; Vulava, 2005), and no harmful effect was not observed at value of rosin is 105-200 mg/kg (Golden, 2006). It was calculated as 57.9 mg/kg for cattle of the same characteristics.

In this study we decided to use SCC over 50.000 as an infected breast. Animal-based fluctuations in SCC were observed during the trial period, which was higher than previously predicted. And the increase in SCC expected with the effect of heat stress could not be seen by experimenting in the summer months. However, the experiment was continued and the differences in the experimental and control groups exposed to the same effects were tried to be observed. As a result of drinking the formulated oral suspension formulation for 33 days, weekly SCC were evaluated with the non-parametric Mann Whitney U test. However, no significant difference was observed in any of the weekly comparisons.

When the formulation applied in the light of the available data is analyzed statistically, it is noteworthy that although there is no significant difference in the amount of SCC and milk in the experimental group compared to the control group, the SCC, milk amounts and milk fat yield the desired results in favor of the experimental group only in the period when the formulation was applied.

In order to support the results obtained, it was expected that the formulation would suppress methanogens which causes energy loss by producing methane in the cattle digestive system according to the rumen volume at the dose used in the in vivo experiment (Sierra-Alvarez and Lettinga, 1990). It can be thought that the increase in the amount of milk in the experiment group compared to the control group in the period when the formulation applicated, decreased the energy loss and increased the amount of milk and supported the milk fat.

Conclusion

Lastly, an oral formulation that include of colophony and turpentine was developed. Although it was not statistically significant at the end of the presented study, it was observed that after the 2nd week, the amount of milk and the amount of fat in the milk increased in the experimental group. SCC decreased more in the experimental group compared to the control group within two weeks also.

It is remarkable as an antibiotic-free method to prevent mastitis by using the oral route and to increase milk yield, as an easy application and an economical solution. Further research is required to elucidate the mechanism of action of this combination based on experimental results. The formulation which has resin consistent has already started to attract attention as an active ingredient that we will see frequently in terms of animal health in the coming years.

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