Abstract—Clove and orange peel oils were used for rumen manipulation in ruminant animal production. However there is limited study with true in vitro rumen digestibility. The objective of this study was to evaluate combination effect of clove and orange peel oils on in vitro digestion of Dairy Total Mixed Ration (TMR) using ANKOM DAISY™ Incubator. Ruminal fluid for in vitro digestion technique was prepared as in vitro digestibility ANKOM method. The results indicated that in vitro true DM disappearance (IVTDMD) and in vitro neutral detergent fiber disappearance (IVNDFD) of dairy TMR were significant (P <0.01) affected by clove, orange peel oils and their combination. Clove increased IVTDMD and IVNDFD and energy estimate (TDN, ME and NEI) of TMR, while orange peel oils decreased. Therefore, there was antagonistic effects between CO and OP 300 ppm when they were used together in combination treatment for decreasing in vitro digestion of dairy TMR.

Index Terms—clove oil, orange peel oil, in vitro digestion, ANKOM daisy™ incubator, TMR.

I. INTRODUCTION

Some essential oils which have been used for animal feed additive may substitute the use of growth promoters such as antibiotics and hormones. Clove and orange peel essential oils were used for rumen manipulation in ruminant animal production. Major component of clove oil were eugenol and b-caryophylene which is a phenolic non-nutrient component which reported that they had effect on all in vitro rumen fermentation products, VFA, N-NH₃ and rumen microbe [1], [2]. Limonene as major component of essential oils in orange peel essential oil was less used as rumen modifier, but it was reported that it could increase dry mater and NDF digestibility doses 50, 100 and 150 ppm with wheat straw as substrate [3]. The combination between essential oils may result in additive and/or synergic effects that may enhance efficiency of rumen microbial fermentation and nutrient utilization in ruminants. The combination of clove and orange peel oils at optimum doses might effect on rumen microbe balance for ruminal digestion.

The digestibility of feeds could be estimated by some methods known as in vitro techniques. In vitro digestibility method was developed by Tilley and Terry [4] has long been regarded as an accurate in vitro method for predicting diet digestibility. It had been modified by the method and in vitro buffer composition [5] and result in modified equipment and reagent that use are in the method [6], [7]. In vitro digestion method was developed for multiple analysis of feedstuff, reducing labour demands and improving the precision of the assay, Daisy™ incubator (ANKOM Technology Corp., Fairport, NY, USA) makes in vitro dry matter disappearance study easy and efficient because it use an equipment which was designed with four rotating digestion jar and maintains constant, uniform heat and agitation within a controlled chamber (preset at 39.5 °C). The filter bag (ANKOM F57) in Daisy™ incubator method was reported gave a more precise prediction of conventionally measured digestibility estimates than the alternative bags [8]. The bags ensure a more standardized, repeatable alternative to the Tilley and Terry method. Because the in vitro rotating jar technique is a simple apparatus, further improvement would probably be obtained by reducing the laboratory differences in rumen collection procedures and type of animal donors, which, however, reflect practical conditions [9].

The objective of this study were to determine the effect of clove oil ppm (CO 300), orange peel oil 300 ppm (OP 300) and their combination (OP 300/CO 300) at 30 hour incubation on rumen in vitro true DM disappearance (IVTDMD) and neutral detergent fiber disappearance (IVNDFD) of dairy total mixed ration (TMR) using ANKOM DAISY™ incubator.

II. MATERIAL AND METHODS
A. Ruminal Fluid Donor Animals

Three ruminally cannulated cows were used and individually penned indoors at Research and Application Farm, Faculty of Agriculture, University of Cukurova, Turkey. Animal was adapted with total mixed ration (TMR) containing 60% concentrate and 40% alfalfa hay sized 1-2 cm for 2 weeks before taking ruminal fluid for inoculum medium at in vitro digestion assay. Ruminal fluids were collected from different sites within the rumen before morning feeding at 08.00 am.

B. Experimental Diets

Experimental diet was total mixed ration containing 60% concentrated feed and 40% alfalfa hay. Alfalfa hay was chopped to be 1.5 - 2 cm size. Nutrients containin alfalfa hay, concentrated feed and TMR were described in Table I. TMR was used for feeding to cannulated cows and used for substrate fermentation in it vitro digestion assay. Alfalfa hay, concentrate, TMR and other feed ingredients which were used for this experiment were assayed for proximate [10], Fiber analysis [7]. Fiber ingredients which were used for this experiment were assay. Alfalfa hay, concentrate, TMR and other feed

After NDF data recorded, the filter bag was placed in Kjehdahl distillation tube for CP analysis as described above (Residual CP). Residual CP was corrected by CP values from blank filter bag.

C. In Vitro Digestion Methods

In vitro digestion method using ANKOM DAISY11 incubator had several steps: 0.25 g substrat TMR preparing in filter bag (F57); buffer solutions, rumen inoculum and essential oils mixing; 30 hours incubation and NDF analysis. The final bag weight after NDF analysis was recorded as final weight \( W_2 \) which its values of samples was used for energy and digestibility estimation. Estimation energy of feed was approached by UC Davis (UCD) factorial [11] using equation 3. In vitro true DM disappearance (IVTDMD) and in vitro NDF disappearance (IVNDFD) were calculated with equation (1 and 2)

\[
IVNDFD \ (% \ DM) = 100 \times \left[ \frac{W_2 \times % \text{NDF}_{\text{Feed}} - (W_1 - (W_1 \times C_I))}{W_2 \times % \text{DM}_{\text{Feed}}} \right] \\
IVTDMD \ (% \ DM) = 100 - \left[ \frac{(W_1 - (W_1 \times C_I)) \times 100}{W_2 \times % \text{DM}_{\text{Feed}}} \right]
\]

where \( W_1 \) is weight of filter bag, \( W_2 \) is weight of sample, \( W_3 \) is final weight (Filter bag + sample), \( \text{NDF}_{\text{Feed}} \) is % of NDF contain in Feed (％DM), \( \text{DM}_{\text{Feed}} \) is % of dry matter contain in feed and \( C_I \) is correction of factor (blank filter bag NDF value).

IVNDF or digestible NDF (dNDF) is used for energy estimation approach by energy equation of UC Davis (equation 3, 4, 5,6) result in TDN, DE, ME and NEL values.

\[
\text{TDN}_{\text{1xM}} = \left( (\text{CP-ADICP}) \times (\text{FT/5}) \times 0.98 \right) \\
+ \left( (\text{CP-ADICP}) \times (1-\text{FT/5}) \times 0.80 \right) \\
+ \left( \text{EE} \times 0.98 \times 2.25 \right) + \left( \text{NDF-dNDF} \right) \\
+ \left( 0.98 \times (100-\text{ASH-EE-NDF-CP}) \right)
\]

\[
\text{DE}_{\text{1xM}} = 0.04409 \times \text{TDN}_{\text{1xM}}
\]

\[
\text{ME}_{\text{1xM}} = (\text{DE}_{\text{1xM}}) \times 1.01 - 0.45
\]

\[
\text{NEL}_{\text{1xM}} = (\text{TDN}_{\text{1xM}}) \times 0.2066 - 0.12
\]

\[
\text{TDN}_{\text{1xM}} \times \text{DE}_{\text{1xM}} \times \text{ME}_{\text{1xM}} \times \text{NEL}_{\text{1xM}}
\]

\[
\text{Discount} = \left( (0.033 + (0.132 \times \text{NDF}(\% \text{DM}))) \right) \\
- \left( 0.033 \times \text{NEL}_{\text{1xM}} \times \text{MCal/(Kg)} \right) \\
+ (\text{NSC}(\% \text{DM}) \times 0.05)
\]

D. Statistical Analysis

Treatments of this experiment were 1) control, 2) Orange peel oil 300 ppm (OP 300), 3) Clove oil 300 ppm (CO 300) and 4) combination between clove oil 300 ppm and orange peel oils 300 ppm (OPCO 300) in ruminal fluid, which were assigned and analyzed in two by two factorial arrangement in a completely randomized design.

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The data was analyzed by using GLM procedure of SAS 9.1.3 for windows statistical package [12].

\[
Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \beta)_{ij} + \epsilon_{ijk}
\]  

(8)  

where \(Y_{ijk}\) is observed value, \(\mu\) is general mean, \(\alpha_i\) is clove essential oils 300 ppm effect, \(\beta_j\) is orange peel oil 300 ppm effect, \((\alpha \beta)_{ij}\) is their combination effect and \(\epsilon_{ijk}\) = standards error.

### TABLE II. IVTDMD, IVNDFD AND ENERGY ESTIMATED VALUES OF 30 HOURS FERMENTED TMR WITH CO, OP AND THEIR COMBINATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CO-0</th>
<th>CO300</th>
<th>SE</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OP-0</td>
<td>OP-300</td>
<td>OP-0</td>
<td>OP-300</td>
</tr>
<tr>
<td>IVNDFD (% DM)</td>
<td>35.67*</td>
<td>20.74*</td>
<td>48.27*</td>
<td>19.36*</td>
</tr>
<tr>
<td>TDN (Mcal/Kg)</td>
<td>56.67*</td>
<td>67.20*</td>
<td>56.15*</td>
<td>1.53</td>
</tr>
<tr>
<td>ME (Mcal/Kg)</td>
<td>2.33*</td>
<td>2.07*</td>
<td>2.54*</td>
<td>2.05*</td>
</tr>
<tr>
<td>NEF (Mcal/Kg)</td>
<td>1.54*</td>
<td>1.39*</td>
<td>1.67*</td>
<td>1.37*</td>
</tr>
<tr>
<td>IVTDMD (%)</td>
<td>75.41*</td>
<td>69.70*</td>
<td>80.23*</td>
<td>69.18*</td>
</tr>
</tbody>
</table>

Where: *CO* = clove oil, *OP* = orange peel oil. Same letter at the same row indicate no difference between treatment (*P > 0.05*).

### III. RESULT AND DISCUSSION

Clove oil 300 ppm (CO 300) was significantly increased (*P <0.01*) in vitro true DM disappearance (IVTDMD), in vitro NDF disappearance (IVNDFD), and estimated energy contents (TDN, ME and NEL) values compared to control using Daisy™ Incubator, while orange peel oil 300 ppm (OP 300) decreased all of parameters values after 30 hour incubation (Table II). CO significantly (*P <0.01*) increased IVNDFD values compare to control after 30 hour incubation (48.27% from 35.67%), while PO was decreased. The value of IVNDFD of CO 300 ppm or OP 300 ppm linearly resulted to same effect of some energy estimated values (TDN, ME and NEL), it was caused by the equation of energy estimated which used IVNDFD 30 hours as a variable. CO 300 ppm increased TDN value 7.73%, ME value 6.72, NEL value 8.44% from control treatment of TMR. OP 300 decreased TDN value 9.15%, ME value 13.03 NEL value 9.74% from control TMR. However, CO 300 ppm was reported had no effect to increase in vitro rumen gas production using Hohenheim gas technique (HGT) after 96 hour fermentation[13] indicated no negative effect on digestibility of insoluble fraction of TMR, because CO at 0.25 ml and 0.50 ml level of extract could inhibit enzyme CMCase, xylanase and acetylenease[14]. Other in vitro fermentation culture also was reported that eugenol oil from doses to 5000 mg/L had effect on all rumen fermentation products while the eugenol in this research is containing 97.26% or close to 300 mg/L [15]. An unpublished experiment reported that OP 300 ppm increased gas production after 48 fermentation using HGT which is containing more in vitro methane gas production refer to ineffective rumen fermentation.

Combination of CO 300 and OP 300 was significant (*P<0.01*) affected on IVTDMD, IVNDFD and some energy estimated values of TMR. The combination result was affected by OP 300 which was decreased IVNDFD, IVTDMD and some energy values significantly (*P<0.05*). There was no synergistic effect of combination but antagonistic effect when CO 300 ppm and OP 300 ppm were used together in in vitro rumen fermentation.

This result might have been a reflection of high doses of orange peel oil 300 ppm which its high doses of main component (Limonene) having highly antibacterial characteristics especially gram-positive bacteria [16], such as Methanobrevibacter ruminantium [17] which is living synergic with rumen cilia protozoa in rumen. Protozoa in rumen was reported have improvement in digestion of lignocellulose due to bacterial and cilia protozoa synergistic effect [18]. High doses of CO 300 ppm had no negative effect on IVNDFD and IVTDMD when it was used alone.

The combination effect of CO 300 and OP 300 showed a negative effect to IVTDMD, IVNDFD and some energy estimated values because of stronger microbial activity of CO 300 and OP 300 while they were used together for 30 hour rumen incubation. Generally, main action mechanism of plant essential oils as rumen manipulator is antimicrobial effects of main component of the essential oils. It was reported that cell membrane disruption would be happen when plant EO’s active with microorganism cell membrane including electron transport, ion gradients, protein translocation, phosphorilation and other enzyme-dependent reactions [19], [20]. The combination effect was not only affected by doses but also with type of essential oil. It was reported that combination of clove oil (800 mg/L) with other essential oil (cinnamaldehyde) had no effects on deaminative activity of rumen bacteria and ammonia N Concentration in vitro rumen fermentation [21] (Benchaar et al 2008), while other combination using less doses of clove oil (90 mg/day and 300 mg/day) affected N metabolism in the rumen by increasing the concentration of small peptide plus amino acid N and decreasing ammonia N concentration [22]. Combination effect of OP 300 with CO 300 ppm increased gas production after 96 fermentation HGT compare to control and single addition of CO and OP. Combination between CO and OP had no negative effect on gas production after 24 hour incubation, which led to an increase in ME of TMR. Menke et al (1988) suggested that GP after 24 hour incubation has a positive correlation with ME in feedstuff. CO 300 ppm had similar effect with the control but did not decrease the ME value of TMR [23].

All of combination between CO and OP had no advantage according to in vitro digestibility because they could reduce methane production but could not keep the
in vitro digestibility to be similar with without essential oil. The result could be explained because of activity of limonene in PO and clove in CO which have methane reducing compounds. There was a synergistic effect of combination for methane reducing but also antagonistic effect of combination for decreasing in vitro digestibility. The Advantage of CO 300 in combination is covering of

The chemical composition of clove and orange peel essential oil samples indicated that eugenol contained in clove oil is 97.26% and limonene contained in orange peel oil is 98.08%. Mostly pure active component of essential oils containing in CO and OP were used in this experiment. In vitro rumen fermentation method using Daisy incubator® (ANKOM) proved that the in vitro true digestibility of DM and NDF could explain estimation of energy values more accurate than other in vitro rumen fermentation method.

IV. CONCLUSION

Clove and orange peel essential oils 300 ppm affected in vitro digestion of dairy TMR. However, there was antagonistic effect on in vitro digestion value while they were used together in in vitro rumen fermentation due to stronger microbial activity of CO 300 ppm and OP 300 ppm.

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REFERENCES


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