Combination Effect of Clove and Orange Peel Oils on in Vitro Digestion of Dairy Total Mixed Ration Using ANKOM DAISY^{II} Incubator

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***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^{II} 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 NEl) 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^{ii} 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-NH_3 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^{II} incubator (ANKOM Techology 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 ^{o}C). The filter bag (ANKOM F57) in Daisy^{II} 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 diaspperance (IVNDFD) of dairy total mixed ration (TMR) using ANKOM DAISY^{II} incubator.

II. Material and Methods

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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) contaning 60% concentrate and 40% alfalfa hay sized 1-2 cm for 2 weeks before taking ruminal fluid for inoculums 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 contain in alfalfa hay, concentrated fed and TMR were described in Table I. TMR was used for feeding to cannulated cows and used for substrate fermentation in in vitro digestion assay. Alfalfa hay, concentrate, TMR and other feed ingredients which were used for this experiment were analysed for proximate [10], Fiber analysis [7]. Fiber analysis for neutral detergent fiber and acid detergen fiber (ADF) were analysed using a heat stable α-amylase at 0,2 ml/g DM. Neutral detergent insoluble N (NDIN) or crude protein CP (NDICP) represents the protein associated with cell wall and insoluble in neutral detergent solution. After NDF data recorded, the filter bag was placed in Kjehdahl destilation tube for CP analysis as described above (Residual CP). Residual CP was corrected by CP values from blank filter bag.

TABLE I. TOTAL MIXED RATION AS EXPERIMENTAL DIET : INGREDIENTS AND ITS CHEMICAL COMPOSITION

	Concentrate (%)	Alfalfa hay (%)	TMR (%)
Ingredients :	60	40	100
Barley grain	9.59		
Corn grain	3.00		
Wheat middlings	3.59		
Wheat brand	4.19		
DGGS	15.32		
Sun flower meal	11.98		
Soybean meal	6.25		
Mineral	1.88		
Salt	0.34		
Molasses	3.59		
Vitamin	0.09		
DCP	0.17		
Chemical composition :			
Dry Matter (%)	89.95	92.80	91.95
OM (% DM)	91.91	91.79	91.70
CP (% DM)	23.56	14.73	20.29
EE (% DM)	2.15	1.15	1.39
CF (% DM)	11.21	36.51	21.33
NFE (% DM)	54.98	39.41	48.69
Ash (% DM)	8.09	8.21	8.30
NDF (% DM)	28.53	60.30	41.23
ADF (%DM)	19.84	49.27	31.61
ADICP (% CP)	10.28	8.54	9.22
NDICP (% CP)	7.20	4.91	8.09
ME (MCal/kg)[1]			2.41

DM = dry matter, OM = organic matter, CP = crude protein, EE = extract ether, CF = crude fiber, NFE = nitrogen free extract, NDF = neutral detergent fiber, ADF = Acid detergent fiber, ADICP = Acid detergent insoluble crude protein, NDICP = Neutral Detergent insoluble crude protein, [1)]metabolizable energy estimated by equation 5.

C. In Vitro Digestion Methods

In vitro digestion method using ANKOM DAISY^{II} 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_3) 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)

$$\text{IVNDFD (\% DM)} = 100 \times [(W_2 \times \%NDF_{Feed}) - (W_3 - (W_1 \times C_1))]/(W_{2\times\%}DM_{Feed}) \quad (1)$$

$$\text{IVTDMD (\%DM)} = \frac{100 - [(W_3 - (W_1 \times C_1)) \times 100]}{(W_2 \times \% DM_{Feed})} \quad (2)$$

where W_1 is weight of filter bag, W_2 is weight of sample, W_3 is final weight (Filter bag + sample), NDF_{Feed} is % of NDF contain in Feed (%DM), DM_{Feed} is % of dry matter contain in feed and C_1 is correction of factor (blank filter bag NDF value).

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

$$TDN_{(1xM)} = ((CP\text{-}ADICP)*(FT/5)*0.98) + ((CP–ADICP)*(1-(FT/5))*0.80) + ((EE\text{-}1)*0.98*2.25) + (NDF*dNDF) + (0.98*(100\text{-}ASH\text{-}EE\text{-}NDF\text{-}CP))) \quad (3)$$

$$DE_{(1xM)} = (0.04409 \times TDN_{(1xM)}) \quad (4)$$

$$ME_{(1xM)} = ((DE_{(1xM)})*1.01) - 0.45 \quad (5)$$

$$NEL_{(1xM)} = ((TDN_{(1xM)})*0.0266) - 0.12 \quad (6)$$

$TDN_{(1xM)}$ is the total digestible nutrient value of a feed or diet at maintenance intake. Because net energy for lactation (NEL) at maintenance $(NEL)_{(1xM)}$ is not representative of the energy value of feed or diet at production level, a discount factor was developed to correct for decrease net energy level at production (NEL, $_{3xM)}$). In UCD factorial approach to estimate feed energy levels. A discount energy factor was formulated based on NDF and non structural carbohydrate (NSC) content of feedstuff and $NEL_{(1xM)}$ values as % per unit of energy intake (M) which it is calculated with equation 7.

$$\text{Discount} = ((0.033 + (0.132*NDF(\%DM))) - (0.033*NEL_{(1xM)},MCal/Kg))) + (NSC(\%DM)*0.05) \quad (7)$$

D. Statistic 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.

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

$$Yijk = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk} \quad (8)$$

where Y_{ijk} is observed value, μ is general mean, αi is clove essential oils 300 ppm effect, βj is orange peel oil 300 ppm effect, $(\alpha\beta)_{ij}$ is their combination effect and e_{ijk} = standards error.

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

Parameter	CO-0		CO300		SE	P Value		
	OP-0	OP-300	OP-0	OP-300		CO	OP	CO*OP
IVNDFD (% DM)	35.67[b]	20.74[a]	48.27[c]	19.36[a]	3.99	0.18	0.01	0.10
TDN_{1xM} (%)	62.38[b]	56.67[a]	67.20[c]	56.15[a]	1.53	0.18	0.01	0.10
ME_{1xM}(Mcal/Kg)	2.33[b]	2.07[a]	2.54[c]	2.05[a]	0.07	0.18	0.01	0.10
NEl_{3xM}(Mcal/kg)	1.54[b]	1.39[a]	1.67[c]	1.37[a]	0.04	0.18	0.01	0.10
IVTDMD (%)	75.41[b]	69.70[a]	80.23[c]	69.18[a]	1.53	0.18	0.01	0.10

Where : CO= Clove oils, OP = orange peel Oils, Same letter at the same row indicate to 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[II] Incubator, while orange peel oils 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 treatmen 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 acetylesterase [14]. Other in vitro fermentation culture also was reported that eugenol oil from doses 3 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 uneffective rumen fermentation.

Combination of CO 300 and OP 300 was significant (P<0.01) affected on IVTDMD, IVNDF and some energy estimated values of TMR. The combination result was affected by OP 300 which was decreased IVNDF, 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 negative effect of 300 ppm OP result in similar effect between combination and control (without essential oil).

The chemical composition of clove and orange peel essential oil samples indicated that eugenol contained in clove oil is 97.26% and limonede 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[II] (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. Conclussion

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

[1] M. Busquet, S. Calsamiglia, A. Ferret, and C. Kamel, "Plant extracts affect in vitro rumen microbial fermentation," *J. Dairy Sci.*, vol. 89, pp. 761-771, 2006.

[2] L. Castillejos, S. Calsamiglia, A. Ferret, and C. Kamel, "Effects of essential oils active compounds on rumen microbial fermentation and nutrient flow in vitro system," *J. dairy. Sci.*, vol. 89, pp. 2649 -2658, 2006.

[3] M. Gorgulu, F. Ozogul, and E. Yildiz, "The effect of some essential oils on in vitro true digestibility of energy, protein, cellulose source of feeds and milk yield and milk composition in high yielding dairy cows," Final Report Project, No. 1070822, TUBITAK, 2010.

[4] J. M. A. Tilley and R. A. Terry, "A 2-stage technique for the in vitro digestion of forage crops," *J. Br. Grassl.Soc.*, vol. 18, pp. 104 -111, 1963.

[5] E. L. McDougall, "Studies on ruminant saliva: Part 1. The composition and output of sheep's saliva," *Biochem. J.*, vol. 42, pp. 99–109, 1948.

[6] H. K. Goering and P. J. Van Soest, "Forage fiber Analysis (apparatus, reagents, procedures and some application)," *Agriculture Handbook*, No. 379. USDA-ARS, Washington, DC., 1970.

[7] P. J. Van Soest and R. H. Wine, "Use of detergent in the analysis of fibrous feed, 'IV. Determination of plant cell-wall constituents'," *J. of the Association of Official Agriculture Chemists*, vol. 50, pp. 50-55, 1967.

[8] A. T. Adesogan, "Effect of bag type on the apparent digestibility of feeds in ANKOM Daisy[II] incubators," *Anim. Feed Sci. Tech.* vol, 119, pp. 333-344, 2005.

[9] M. Spanghero, *et al.*, "Technical note: Precision and accuracy of in vitro digestion of neutral detergent fiber and predicted net energy of lactation content of fibrous feeds," *J. Dairy Sci.*, vol. 10, pp. 4899-9, 2010.

[10] AOAC, Association of official Analytical Chemist, *Official Methods of Analyses*, ed. 12th., Washington DC., 1998.

[11] P. H. Robinson, D. D. Givens, and G. Getachew, "Evaluation of NRC, UC Davis and ADAS approaches to estimate the metabolizable energy values of feeds at maintenance energy intake from equation utilizing chemical assays and in vitro determinations," *J. Anim. Feed. Sci. & Tech*., vol. 114, pp. 75-90, 2004.

[12] SAS Institue Inc., *SAS 9.1.3. Procedures Guide*, 2nd Ed., vol. 1, 2, 3 and 4, Cary, NC.

[13] M. N. Rofiq, S. Martono, M. Gorgulu, and M. Boga, "Combination effect of clove and cinnamon oil on in vitro rumen gas and methane production," in *Proc. 2nd International Seminar on Animal Industry*, Jakarta, 2012, pp. 431-437.

[14] A. K. Patra, "Meta-analyses of effects of phytochemicals on digestibility and rumen fermentation characteristics associated with methanogenesis," *J. Sci. Food. Agric.*, 2010.

[15] M. Busquet, S. Calsamiglia, A. Ferret, P. W. Cardozo, and C. Kamel, "Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation continuous culture," *Anim. Feed Sci. Tech.*, vol. 123, pp. 597-613, 2005.

[16] R. Dabbah, V. M. Edwards, and W. A. Moats, "Antimicrobial action of some citrus fruit oils on selected food-borne bacteria," *Appl. Microbiol.*, vol. 19, pp. 27-31, 1970.

[17] P. H. Smith and R. E. Hungate, "Isolation and characterization of Methanobacterium ruminantium n. sp.," *Journal of Bacteriology*, vol. 75, pp. 713-718, 1985.

[18] J. P. Jouany and J. Senaud, "Role of rumen protozoa in digestion of food cellulosic materials," *Ann rech. Vet.*, vol. 10 no. 2, pp. 261-263, 1979.

[19] A. Ultee, M. Bennik, and R. Moezelaar, "The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus," *App. Env. Mic.*, vol. 68, pp. 1561-1568, 2002.

[20] H. J. D. Dorman and S. G. Deans, "Antimicrobial agents from plants: antibacterial activity of plant volatile oils," *J. Appl. Microbiol*, vol. 88, pp. 308-316, 2000.

[21] C. Benchaar *et al.*, "A review of plant-derived essential oils in ruminant nutrition and production," *J. Anim Feed Sci.and Technol.*, vol. 145, pp. 209-228, 2008.

[22] P. W. Cardozo, S. calsamiglia, A. Ferret, and C. Kamel, "Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed high concentrate diet," *J. Anim. Sci.*, vol. 83, pp. 2572-2579, 2006

[23] K. H. Menke and H. Steingass, "Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid," *Animal Research and Development*, vol. 28, pp. 7-55, 1988.

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