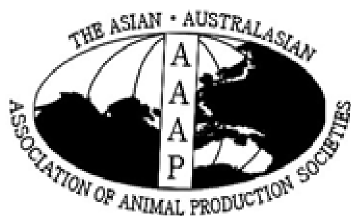

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Supplementation of Essential Oil Extracted from Citrus Peel to Animal Feeds Decreases Microbial Activity and Aflatoxin Contamination without Disrupting *In vitro* Ruminal Fermentation

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ABSTRACT : Long-term storage of feeds or feedstuffs in high temperature and humid conditions can be difficult because of microbial contamination. Essential oil isolated from industrial waste citrus peel could be used as a preservative because it is likely to have anti-bacterial and anti-fungal activity. Our objective was to determine whether different levels (0.028, 0.056 and 0.112 g/kg) of citrus essential oil (CEO) would provide anti-microbial activity and enhance preservation of animal feed without influencing rumen fermentation. At 0.112 g/kg, CEO inhibited growth of *Escherichia coli* (ATCC 25922) and *Salmonela enteritidis* (IFO 3313). Growth of *E. coli* recovered after 24 h of incubation, but *S. enteritidis* continued to be inhibited for 72 h. Preservation of antibiotic-free diets for swine was assessed by observing anti-aflatoxin activity. Aflatoxin was detected in control feed samples on days 16 (8 ppb) and 21 (8 ppb) and in anti-fungal agent (AA) treated samples on days 16 (2 ppb) and 21 (4 ppb). However, aflatoxin was not detected in feed samples treated with CEO. Treatment with CEO and AA did not influence ruminal pH, dry matter digestibility (DMD) or organic matter digestibility (OMD) over 48 h of incubation in rumen fluid. Acetate and propionate were slightly higher with CEO treatment ($p<0.05$), but total concentration of volatile fatty acid (VFA) was not significantly affected by treatment. Ammonia-N concentration was slightly higher for the control treatment ($p<0.05$). This study showed that treating feed with CEO enhances preservation of animal feed without influencing *in vitro* rumen fermentation. (**Key Words :** Citrus Essential Oil, Feed Preservation, Antimicrobial Activity, Rumen Fermentation)

INTRODUCTION

Long-term storage of animal feeds in high humidity and high temperature conditions is a problem because feeds can be decomposed easily by harmful microorganisms. *Aspergillus* and other toxic metabolite fungi produce aflatoxins, and food poisoning bacteria, such as *S. enteritidis* or *E. coli*, in feeds or feedstuffs for animal consumption are extremely dangerous for human and animal health (Cole and Cox, 1981). Diets for pig are mainly consisted of cereal grains. Those cereal grains are easily contaminated by aflatoxin-producing molds. Pig is one of the most sensitive animal to the effects of aflatoxin, therefore aflatoxin contaminated feed seriously may affect the swine industry (Shi et al., 2005).

Essential oils (EO) are natural components of a variety of foods and beverages and are found in many fruits, vegetables, and meats (Marshall, 1995). The major component of EO in citrus fruits and grapefruit is limonene (Kesterson et al., 1971). Citrus fruits yield high amounts of EO when extracted using steam distillation or cold-pressing methods (Caccioni et al., 1998; Choi and Sawamura, 2000). The EO has biological activities such as anti-microbial, allelopathic, antioxidant and bioregulatory properties (French, 1985; Elakovich, 1988; Deans, 1991; Caccioni and Guizzardi, 1994; Caccioni et al., 1995). Effects of EO supplements on rumen fermentation have been reported with diets for cows and sheep (Molero et al., 2004; Newbold et al., 2004). However, there has been no report on effects of EO extracted from citrus peel (CEO) on either biological activity for preservation of feeds or feedstuffs, or on rumen fermentation. The aim of this study was, therefore,

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Table 1. Ingredients of antibiotic free-diet for swine

Item	Content
Ingredients [a]	
Yellow corn	65.16
Rice hull	8.00
Soybean meal	19.00
Lupin seed	3.20
Animal fat	3.00
Molasses	3.00
Limestone	0.90
Salt	0.30
Hog premix[b]	0.30
Methionine	0.01
Lysine	0.04

[a] Values are expressed as % of dietary DM.

[b] Hog premix contains the following per kilogram: vitamin A, 12,000,000 IU; vitamin D_3, 2,000,000 IU; vitamin E, 35,000 mg; vitamin K_3, 3,300 mg; vitamin B_2, 3,000 mg; vitamin B_{12}, 33,000 μg; vitamin C, 40,000 mg; Pantothenic acid, 20,000 mg; Niacin, 30,000 mg; Biotin, 100,000 μg; $FeSO_4$, 73.500 mg; $ZnSO_4$, 56,000 mg; $MnSO_4$, 15,750 mg; $CuSO_4$, 86,100 mg; $Ca(IO_3)_2$, 175 mg; S, 17,500 mg; $CoSO_4$, 157 mg; Na_2SeO_3, 105 mg.

to investigate the anti-microbial activity of CEO on antibiotic-free feed for swine using tube dilution, agar diffusion (to test anti-bacterial activity) and preservation testing (to test anti-fungal activity), and to investigate the effects of CEO on *in vitro* rumen fermentation.

MATERIALS AND METHODS

Collection of citrus peel

Industrial waste citrus peel was collected from citrus juice manufacturers in the southern region of Korea. Essential oil was extracted by steam distillation and solvent extraction. The CEO was collected in sealed glass containers and refrigerated in the dark at 4°C until used.

Distillation procedure

Citrus peel was ground to pass through a 5-mm screen using a Wiley Mill (DB-M1103, Myung Sung, Korea). Milled samples (Approx, 400 to 500 g) were subjected to steam distillation (Caccioni et al., 1998) at about 110°C until there was no further increase in the volume of CEO collected. After distillation, residual citrus peel was placed in 70% methanol at ambient temperature (about 26 °C) for 4 days. After filtration through Whatman No. 1 filter papers, distilled CEO and methanol-extracted CEO were mixed. Finally, All methanol was evaporated under vacuum condition and 100% of CEO was applied in this study.

Anti-bacterial activity of antibiotic-free feed for swine

Bacteria strains and culture conditions : Major human and animal pathogens, *E. coli* (ATCC 25922) and *S. enteritidis* (IFO3313) were obtained from the culture collection of the Food Science Department in Hankyong National University. These bacteria were cultured in MRS broth media (Difico, Korea) and incubated for 24 h at 37°C. Incubated bacteria were plated using a sterilized glass stick onto the surface of MRS agar media (Difico, Korea) slants and incubated for 24 h at 37°C. Anti-bacterial tests were carried out by a disc diffusion method (Murray et al., 1995) using 100 μl of bacterial suspension containing 10^8 CFU/ml.

Disc-diffusion assay : 0.028 (500 ppm), 0.056 (1,000 ppm) and 0.112 (2,000 ppm) g/kg of CEO were dissolved in methanol and sterilized by filtration through 0.45 μm filters (Millipore, Korea). Discs of sterilized filter paper (Whatman, Korea), 6 mm in diameter, were impregnated with the CEO solutions and placed on MRS agar media. A negative control was prepared using methanol. Inoculated plates were incubated at 37°C for 24 h. Anti-bacterial activity was evaluated by measuring the zone of inhibition.

Tube-dilution assay : Inoculates of the bacterial strains were prepared from 24 h MRS broth media cultures. Different concentrations (0.028, 0.056 and 0.112 g/kg) of CEO in methanol were added to 10 ml bacteria culture tubes. The culture tubes were then incubated at 37°C for 12, 24, 36, 48, 60, and 72 h. Bacterial growth at each incubation time was measured using a UV spectrophotometer (620 nm) (UV-160, Shimadzu, Japan).

Anti-aflatoxin activity of antibiotic-free feed for swine

Aflatoxin assay and sample preparation : Experimental samples (Table 1) were ground to pass through a 2-mm screen using a Wiley Mill and sterilized at 121°C for 15 min. CEO was dissolved in methanol to give final concentrations of 0.028, 0.056 and 0.112 g/kg. These CEO solutions were then gently sprayed onto feed samples. Three samples (15 g) of each treated feed were placed on sterilized petri-dishes and incubated at 30±0.5°C for 0, 3, 6, 9, 16, and 21 days. Humidity was maintained between 78% and 83%. Samples treated with 0.05 g/kg AA, containing 62% propionic acid, 5% acetic acid, 1% sorbic acid, 1% benzoic acid and 1% phosphoric acid, (Jeong Green, Korea) were used as positive controls and samples treated with methanol were used as negative controls. Samples of feed were collected after 16 and 21 days. Aflatoxin concentrations were measured using Aflatoxin kits (A-6636 and A-9887, Sigma, Korea), following the manufacturer's instructions.

In vitro ruminal fermentation study

Animal, inocula and substrates : Rumen fluid was collected from a ruminally fistulated Korean cow (Hanwoo) fed individually twice-daily (08:00 and 17:00) a diet containing rice straw and 6kg of a commercial concentrate for more than 5weeks. Samples of rumen contents were taken and transferred to the laboratory in a water bath preheated to 39±0.5°C, squeezed twice through four and eight layers of cheese cloth, purged with CO_2 and mixed

Table 2. Ingredients and chemical composition of the dietary treatment

	Content
Ingredients [a]	
Wheat	3.1
Wheat bran	6.9
Corn gluten feed	4.4
Cotton seed meal	3.7
Coconut meal	1.1
Soy bean meal	18.1
Lime stone	1.2
Lupin	1.2
Corn flaked	30.5
Salts	0.6
Cotton hulls	6.2
Alfalfa cube	2.4
Rice straw	5.0
Alfalfa pellet	2.2
Beet pulp	7.3
Alfalfa hay	6.1
Chemical composition [b]	
Dry matter	88.9
Ether extract	3.73
Crude protein	16.6
Crude fibre	18.57
Ash	8.60

[a, b] Values are expressed as % of dietary DM.

Table 3. Effect of citrus essential oil (CEO) extracted from citrus peel on antibacterial activity using the agar diffusion technique

Microorganism	Concentration of CEO (g/kg)		
	0.112	0.056	0.028
E.coli	+	+	-
Salmonella	++	+	-

Antimicrobial activity indicated as clear zone diameter (Ø): ++, Ø>20 mm; +, Ø = 10-20 mm; -, no clear zone.

with McDougall buffer (McDougall, 1948) at the ratio of 1:1. Aliquots (200 ml) of fluid (100 ml buffer+100 ml rumen fluid) were dispensed by an automatic pump into 250 ml serum bottles containing 4 g of feed substrate. The bottles were capped with butyl-rubber stoppers containing gas regulators and placed in a shaking incubator at 39±0.5°C, 100 rpm. Samples were incubated for 48 h. Feed substrates were prepared by grinding a control diet (Table 2) to pass through a 2-mm screen using a Wiley Mill. Milled feed was discarded if it passed through a 250 μm sieve. Test feed substrate samples were prepared by gently spraying samples of the control diet with 0.05 g/kg AA or 0.112 g/kg CEO.

Chemical analysis

The pH of fermented samples was determined by the method of Briggs et al. (1957). Cell wall content was estimated by the method of Goering and Van Soest (1970). *In vitro* DMD and OMD were determined by AOAC (1990) methods. VFA in samples of supernatant fluid were

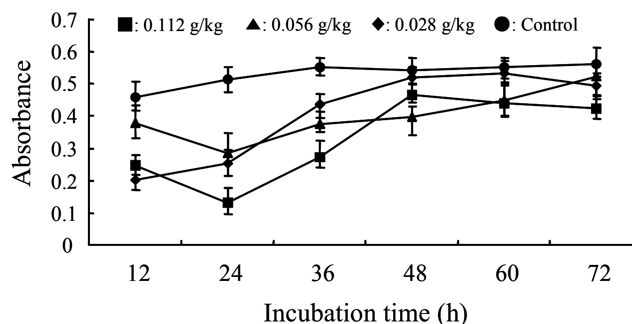

Figure 1. Effect of citrus essential oil extracted from citrus peel on antibacterial activity against *Escherichia coli* using the tube dilution technique.

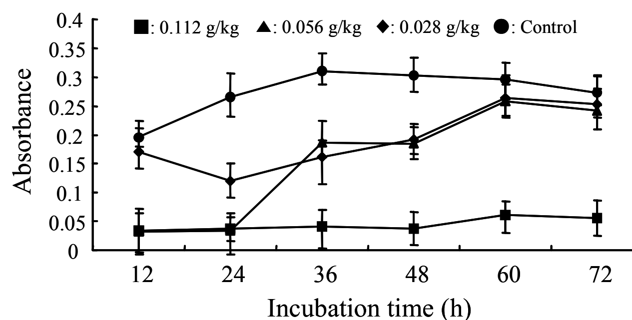

Figure 2. Effect of citrus essential oil extracted from citrus peel on antibacterial activity against *Salmonella enteritidis* using the tube dilution technique.

measured with a gas chromatograph (Hewlett Packard 6890, USA; column temperature: 120°C; injector temperature: 265°C; detector temperature: 240 °C) equipped with an autosampler and a cross-linked polyethylene glycol, 0.53 mm×30 m FFAP column (Hewlett Packard, USA). Ammonia was measured by a colorimetric method (Chaney and Marbach, 1962).

Statistical analysis

Statistical analysis was carried out using the Statistical Analysis System (SAS Version 6.12, 1996). Treatment and incubation time effects on CEO in each culture were tested by analysis of variance. The differences of means between treatments were compared by Duncan's multiple range test, using General Linear Model (GLM) procedures of SAS package.

RESULTS AND DISCUSSION

Anti-bacterial activity

The CEO obtained from citrus peel was yellow in colour and had observed antibacterial activities (Table 3, Figures 1 and 2). Under the conditions of this study, maximum anti-microbial or aflatoxin activity was found to be 0.112 g/kg in both disc-diffusion assay and tube-dilution

Table 4. Aflatoxin content (ppb) of antibiotic-free diet samples for swine measured at 16 and 21 days of storage

Storage (d)	Treatment (g/kg)				
	Control	AA[a] 0.05	CEO[b] 0.028	CEO 0.056	CEO 0.112
16	8	2	nd[c]	nd	nd
21	8	4	nd	nd	nd

[a] AA: Antifungal agent treatment.
[b] CEO: Citrus essential oil. [c] nd: Not detected.

assay. Growth of *E. coli* recovered after 24 h incubation, but *S. enteritidis* continued to be inhibited until the end of incubation (Figures 1 and 2). Farber and Peterkin (1991) noted that *E. coli* can grow in various environmental conditions because they have more ability to adapt, even at low pH between 2 and 4 than any other harmful bacteria. This might be the reason why *E. coli* recovered after 24 h in the present study.

Norman et al. (1967) and McCallet and Torres-Grifol (1992) demonstrated that an injured orange releases a much greater amount of terpene peel-oil constituents than healthy fruits. This might be the source of anti-bacterial activity found in CEO. In the present study, we only tested CEO against the most harmful Gram-negative bacteria because Gram-positive bacteria are more sensitive to EO plant extracts than Gram-negative bacteria (Cosentino et al., 1999; Karaman et al., 2003; de Carvaho Jr et al., 2004). Our results were in agreement with the study by Alderman and Marth (1976), who reported the anti-microbial activity of CEO and limonene from citrus oil.

Anti-aflatoxin activity

Aflatoxins are extremely toxic and secondary metabolites produced by *Aspergillus*, specifically *A. flavus* and *A. parasiticus*, which are found worldwide in air and soil, and are also found in biologically contaminated food or feed. Aflatoxins are strong hepatoxins and are internationally classified as carcinogenic compounds that have been implicated as causative agents in human hepatic and extra-hepatic carcinogenesis (Massey et al., 1995). Therefore, suppression of aflatoxin-producing fungi is very important for feed and food industries. Aflatoxin was measured in samples stored for 16 and 21 days that had previously been used for measuring colour change (Table 4). At 16 and 20 days of storage, aflatoxin content was 8 ppb in the control samples. In the AA treated samples aflatoxin content was 2 ppb after 16 days of storage and increased to 4 ppb at 21 days. No aflatoxin was detected in samples treated with CEO. Antimicrobial activities (anti-bacterial, anti-fungal and anti-yeast activity) have been reported for EO from various plants or fruits, including laiatae, salvia and cordia (Duru et al., 2004; de Carvalho et al., 2004; Tepe et al., 2005). Various components of CEO may act synergistically and several compounds might have stimulating actions on fungal spore germination (French, 1985). There are several reports on the anti-microbial action of CEO (Murdock and Allen, 1960; Subba et al., 1967). These reports demonstrated that the fungi were more resistant than yeasts and bacteria. Karapinar (1985) reported that *A. parasiticus* growth and aflatoxin production were suppressed when CEO was supplemented. Our results showed that CEO has a strong inhibitory effect on biological contamination during the storage of feeds (21 days). The anti-microbial actions of CEO could also be interesting for applications in the feed, food and cosmetics industries.

Table 5. Effects of addition of citrus essential oil (CEO), extracted from citrus peel, and antifungal agent (AA) on pH, DM digestibility and OM digestibility measured by *in vitro* fermentation

	Incubation time (h)					
	3	6	9	12	24	48
pH						
Control[1]	5.70	5.58[a]	5.43	5.45	5.39	5.45
AA[2]	5.47	5.56[a]	5.50	5.44	5.38	5.45
CEO[3]	5.67	5.47[b]	5.49	5.46	5.34	5.29
SEM	0.07	0.03	0.02	0.01	0.02	0.05
DM digestibility[3]						
Control	43.48	36.68	47.50	55.12	59.57	69.09
AA	38.11	43.70	54.64	53.15	62.05	68.23
CEO	42.72	52.07	58.52	57.73	62.14	68.59
SEM	1.68	4.45	3.23	1.33	0.84	0.25
OM digestibility[3]						
Control	15.56[b]	22.68	29.77	41.16	48.80	61.28
AA	15.34[b]	20.24	31.14	39.96	53.81	58.85
CEO	19.34[a]	22.98	29.11	39.42	50.29	59.09
SEM	1.30	0.87	0.60	0.51	1.49	0.77

[a, b] Means with different superscripts in the same row are significantly different ($p<0.05$).
[1] Antibiotic free feed for beef cattle (control).
[2] 0.05 g/kg of antifungal agent (AA).
[3] 0.112 g/kg of essential oil isolated from citrus peel (CEO).

Table 6. Effect of citrus essential oil (CEO), extracted from citrus peel, and antifungal agent (AA) on VFA and ammonia-N concentrations during *in vitro* rumen fermentation

Incubation (h)	Control	AA 0.05 g/kg	CEO 0.112 g/kg	SEM
Acetate (mM)				
3	23.78[ab]	20.14[b]	27.18[a]	2.03
6	24.66[ab]	23.36[b]	26.89[a]	1.03
9	25.76	22.27	28.44	1.79
12	26.37	22.24	28.64	1.87
24	23.70	27.38	27.73	1.29
48	25.78[b]	29.17[a]	26.95[ab]	0.99
Propionate (mM)				
3	7.63[ab]	6.27[b]	8.67[a]	0.69
6	7.91[b]	7.84[b]	9.38[a]	0.50
9	8.12[b]	8.40[b]	10.32[a]	0.69
12	9.77	8.46	10.92	0.71
24	10.80	10.91	11.88	0.34
48	9.71[b]	11.94[a]	11.39[a]	0.67
Butyrate (mM)				
3	3.63[ab]	3.03[b]	3.88[a]	0.25
6	4.04[b]	4.02[b]	4.45[a]	0.14
9	4.54[ab]	4.30[b]	4.90[a]	0.17
12	4.74[a]	4.39[b]	4.45[b]	0.11
24	5.32	5.33	5.20	0.04
48	5.62	5.75	5.61	0.05
Total VFA (mM)				
3	36.42[ab]	30.61[b]	41.05[a]	3.02
6	35.23[b]	36.69[b]	42.25[a]	2.14
9	39.99	35.62	43.68	2.33
12	42.36	36.95	45.63	2.53
24	40.55	46.29	47.32	2.11
48	43.71[b]	50.21[a]	47.35[ab]	1.88
Acetate-propionate ratio (mol/mol)				
3	3.11	3.21	3.12	0.03
6	3.11	2.86	3.08	0.08
9	2.05	2.77	2.59	0.22
12	2.76	2.63	2.68	0.04
24	2.40	2.53	2.33	0.06
48	2.65[a]	2.44[b]	2.37[b]	0.08
NH_3-N (mg/l)				
3	7.22	5.92	6.63	0.38
6	9.66	8.82	9.47	0.25
9	14.18[a]	12.93[ab]	11.37[b]	0.81
12	16.73[a]	14.45[b]	14.28[b]	0.79
24	24.16	22.93	21.97	0.63
48	36.77[a]	30.61[b]	32.56[ab]	0.80

[a, b] Means with different superscripts in the same row are significantly different (p<0.05).

In vitro ruminal fermentation

Our previous *in vitro* study indicated that the anti-bacterial and anti-aflatoxin action was higher when CEO supplemented at 0.112 g/kg. That was why the application of CEO supplemented feed at that level (0.112 g/kg) for ruminants was investigated. The effects of CEO and AA on ruminal pH, DMD and OMD during *in vitro* fermentation were measured (Table 5). Average initial pH was 6.34. In general, addition of CEO or AA solution to the diet did not affect pH, DMD or OMD. However, for the diet containing CEO, pH was lower at 6 h and OMD was higher at 3 h of incubation ($p<0.05$), compared with control and AA treatments. Recently, Molero et al. (2004) studied *in situ* rumen fermentation using a specific blend of EO compounds and also found little or no effect on DMD. CEO treatment tended to increase concentrations of acetate, propionate, butyrate and total VFA over the first 6 to 9 h of fermentation, compared with controls; CEO treatment consistently produced higher VFA concentrations than AA treatment over the first 6 h of fermentation. Neither CEO nor AA treatment affected acetate/propionate (A/P) ratio. CEO treatment lowered ammonia-N concentration compared with control at 9, 12 and 48 h. Newbold et al. (2004) reported lower ammonia-N concentration at 2 h after morning feeding and higher ammonia-N concentration at 6 h after morning feeding in sheep supplemented with 110 mg EO per day compared with control sheep. Ammonia-N production can differ between *in vitro* and *in vivo* studies because of the contribution of rumen recycled nitrogen (Vallimont et al., 2004). Wallace et al. (2002) reported that EO had no influence on VFA or NH_3 concentrations when sheep received 40% concentrate and 60% grass silage with 100 mg EO per day. Newbold et al. (2004) found a similar result with feeding sheep 40% concentrate and 60% forage (grass silage) with 110 mg EO per day, but total VFA and some VFA concentrations were higher (not significantly) in the EO treated group than the control group. A similar trend was found in the current study.

CONCLUSIONS

The results of this study indicate that CEO can inhibit growth of bacteria and fungi on animal feed, thereby prolonging the storage life and safety of feedstuffs. This could be particularly beneficial in hot and humid conditions where feeds are more susceptible to microbial attack. Under the conditions of the current study, maximum anti-microbial or aflatoxin activity was at 0.112 g/kg. *In vitro* studies of rumen fermentation indicate that CEO does not reduce the feeding value of treated feed.

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