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Effects of Freeze-dried Citrus Peel on Feed Preservation, Aflatoxin Contamination and *In vitro* Ruminal Fermentation

I. S. Nam[1, 2], P. C. Garnsworthy[3] and J. H. Ahn[1, *]

[1] Department of Dairy Science, College of Agriculture and Life Sciences, Hankyong National University, Ansong, 456-749, Korea

ABSTRACT : The objective of this study was to investigate antimicrobial activity, during the storage period, of animal feed and any effects on *in vitro* rumen digestion by supplementing different levels (5.55, 11.1, and 22.2 g/kg) of freeze dried citrus peel (FDCP) to the feed compared to untreated feed and feed treated with an antifungal agent (AA) at 0.05 g/kg. In a preservation test, feed supplemented with FDCP showed no deterioration over 21 days. Untreated feed and AA-treated feed, however, showed signs of deterioration after 16 days storage. Yellow colour and red colour, measured by spectro chromameter, decreased in the untreated and AA-treated feeds, but not in feed supplemented with FDCP. Aflatoxin was detected in untreated and AA-treated feeds at 16 days (8 ppb and 2 ppb) and 21 days (8 ppb and 4 ppb), but aflatoxin was not detected in the feed supplemented with FDCP. In a second experiment, fermentation by rumen microorganisms of FDCP (22.2 g/kg) and AA (0.05 g/kg) supplemented feeds was studied *in vitro*. Feeds were incubated with buffered rumen fluid for 3, 6, 9, 12, 24, and 48 h. Dry matter digestibility (DMD) and organic matter digestibility (OMD) were affected by treatment, but ammonia-N, total, and individual volatile fatty acids (VFA) were not adversely affected by treatment. In conclusion, the results indicated that FDCP might be useful for inhibiting microbial growth of animal feed during storage without disrupting rumen fermentation. (**Key Words :** Antimicrobial Activity, Citrus Peel, Aflatoxin, Rumen Digestion)

INTRODUCTION

Large amounts of citrus fruits are produced worldwide for use in industrial products such as juices, ice creams, sweets and snacks. The large quantity of citrus peel (industrial waste) from the citrus industry presents a potential pollution problem, which would be reduced if the waste could be utilised for animal feed. Ashbell and Donahaye (1984) reported that about 50% (by weight) of orange peel waste comprises whole fruit. Silva et al. (1997) and Karabulut et al. (2007) suggested that citrus peel waste and citrus tree leaves could be used for ruminant feed. Broderick et al. (2002) and Hatfield and Weimer (1995) found that supplementation with citrus pulp could increase rumen fermentation rate, improve utilization of non-protein nitrogen through stimulation of microbial protein synthesis, and increase ruminal acetate to propionate ratio without depressing ruminal pH. Natural citrus pulp contains large amounts of pectins and carbohydrates (Rihani et al., 1986), and small amounts of nitrogen (Lanza, 1982). The composition of citrus peel is similar to that of citrus pulp, except citrus peel has a higher content of citrus essential oils (CEO). The CEO in citrus peel have antimicrobial and antioxidant properties (Elakovich, 1988; Deans, 1991; Caccioni and Guizzardi, 1994; Caccioni et al., 1995; Nam et al., 2006), so citrus peel could act as a preservative, which would be beneficial for long term storage of feed. However, most studies of dried citrus peel or pulp for ruminants have used heat treatment to dry the peel. Heat treatment volatilises CEO, so there might not be enough CEO remaining to inhibit aflatoxins and harmful microbes. Freeze drying is an alternative method for drying citrus peel that should retain CEO, but the preservative properties of freeze dried citrus peel are unknown. Moreover, most animal feeds in Asian countries contain chemical antimicrobial agents to increase preservation time by reducing microbial growth without influencing microbial contamination. Chemical antimicrobials might pose

* Corresponding Author: Jong Ho Ahn. Tel: +82-31-670-5124, Fax: +82-31-670-5238, E-mail: jhahn@hknu.ac.kr

[2] Korea Livestock HACCP Accreditation Service, Anyang, 430-731, Korea.

[3] Division of Animal Sciences, University of Nottingham, School of Biosciences, Sutton Bonington Campus, Loughborough, LE12 5RD, UK.

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Table 1. Composition of antibiotic-free diet for swine used in preservation tests

Item	Content
Ingredients [a]	
Yellow Corn	62.27
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
Vit-Min. premix [b]	0.28
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.

problems for human and animal health, and can also interfere with microbial activity during rumen digestion.

The objective of this study was to evaluate the antimicrobial properties of FDCP when used as a feed additive in typical commercial diets. To achieve this objective, two experiments were conducted. The first experiment was designed to test the ability of FDCP to preserve a swine feed with a high cereal-grain content, which was expected to be susceptible to contamination by aflatoxin-producing moulds under warm humid conditions. The second experiment was designed to assess the effects of FDCP on *in vitro* rumen fermentation characteristics when added to a feed with a higher fiber content, which would favor rumen cellulolytic bacteria that are susceptible to antimicrobial agents. Together, therefore, these experiments would indicate both the positive and negative potential of any antimicrobial properties of FDCP.

MATERIALS AND METHODS

Preservation test

Citrus peel (*Rutaceae, Citrus junis*, 5 kg, approximately) was collected from a citrus juice manufacturing factory in southern Korea and dried for 3 to 5 days (Pressure: 2×10^{-4} Torr; Temp: -60°C) in a freeze drier (FD-5512, Il shin, Korea). The citrus peel was then ground using a 2 mm screen Wiley Mill to produce FDCP (DM: 98.57%, Fat: 4.23%, CP: 16.19%, CF: 18.45%, Ash 8.38%). Batches of test feed were prepared by adding FDCP to an antibiotic-free feed for swine (Table 1) and grinding the mixture through a 2 mm screen Wiley Mill. Rates of inclusion of FDCP were 5.55, 11.1 and 22.2 g/kg. A negative control batch of feed was prepared without addition of FDCP and a positive control was prepared by treating feed with a commercial anti-fungal agent (AA) containing 62% propionic acid, 5% acetic acid, 1% sorbic acid, 1% benzoic acid and 1% phosphoric acid (Jeong Green International, Korea) at the manufacturer's recommended rate of 0.05 g/kg feed.

Fifteen g of feed samples were placed in 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%. After each incubation period, colour values (L*; Lightness, a*; redness, b*; yellowness) were measured, to observe which specific colours in samples were changing, using a Spectro chromameter (CM 5081, Minolta). Aflatoxin content of incubated feed batches was determined after 16 and 21 days incubation using Aflatoxin kits (A-6636 and A-9887, Sigma), following the manufacturer's instructions.

In vitro ruminal fermentation test and feed samples

Rumen fluid was collected from a ruminally-fistulated Korean cow (Hanwoo) fed twice daily (08:00 and 17:00) on a diet containing rice straw and 6 kg of a commercial concentrate for more than 5 weeks. Rumen fluid samples were squeezed through four layers and then eight layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. The flask was incubated without disturbance for 30 min in a 39±0.5°C water bath to permit feed particles to rise to the top of the flask.

Particle-free rumen fluid was anaerobically mixed (1:1 ratio) with a buffer (McDougall, 1948) containing 7.5 g $NaHCO_3$, 0.824 g anhydrous Na_2HPO_4, 0.31 g anhydrous KH_2PO_4, 0.03 g $MgSO_4{\cdot}7H_2O$, 3.25 mg anhydrous $CaCl_2$, 2.5 mg $MnCl_2{\cdot}4H_2O$, 0.25 mg $COCl_2{\cdot}6H_2O$ and 2.0 mg $FeSO_4{\cdot}7H_2O$ per litre, and 200 ml was anaerobically transferred to 250 ml bottles containing 4 g of feed samples. Bottles were capped with butyl-rubber stoppers fitted with gas regulators, placed in a shaking incubator (Vision, Korea) at 39±0.5°C, 100 rpm, and incubated for 3, 6, 9, 12, 24 and 48 h.

Feed samples were prepared by grinding a ruminant diet (Table 2) to pass through a 2-mm screen using a Wiley Mill. Milled feed was sieved and material smaller than 250 μm was discarded. Feed samples were supplemented with 22.2 g/kg FDCP, treated with 0.05 g/kg AA (positive control), or left untreated (control).

Chemical analysis

Samples of fluid (50 ml) were removed through the butyl rubber stopper using a 60-ml syringe at 3, 6, 9, 12, 24 and 48 h of incubation. Samples were immediately centrifuged (14,000×g at 4°C for 15 min), and the supernatant stored at -20°C. pH of samples was determined

Table 2. Chemical and ingredient composition of ruminant diet used for *in vitro* rumen fermentation tests

Item	Content
Ingredients[1]	
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
Limestone	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
Sugar beet pulp	7.3
Alfalfa hay (long)	6.1
Chemical composition [2]	
DM	88.9
EE	3.73
CP	16.6
CF	18.6
Ash	6.8

[1, 2] Values are expressed as % of dietary DM.

by the method of Briggs et al. (1957). *In vitro* dry matter digestibility (DMD) and organic matter digestibility (OMD) were determined as described by Moor and Mott (1975) and Laster et al. (2005). VFA in samples of supernatant fluid were measured by gas chromatography (Hewlett Packard 6890, equipped with auto sampler and cross-linked polyethylene glycol, ø0.53 mm×30 m size FFAP column; column temperature: 120°C, injector temperature: 265°C, detector temperature: 240°C). Ammonia-N 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 pH and digestibility of cultures were tested by analysis of variance. Differences between treatment means were compared by Duncan's multiple range test, using General Linear Model (GLM) procedures of the SAS package. All incubations were performed in triplicate.

RESULTS AND DISCUSSION

Preservation test

Aflatoxins are 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 extremely toxic, being 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 important for feed and food industries.

Diets for swine and poultry, and concentrate feeds for ruminants, consist mainly of cereal grains. These cereal grains are easily contaminated by aflatoxin-producing moulds. The pig is one of the most sensitive animals to the effects of aflatoxin, therefore aflatoxin-contaminated feed may seriously affect the swine industry (Shi et al., 2005). Treatment with FDCP enhanced preservation of the antibiotic-free diet for swine by preventing microbial activity (Figure 1). Untreated (Control) feed samples

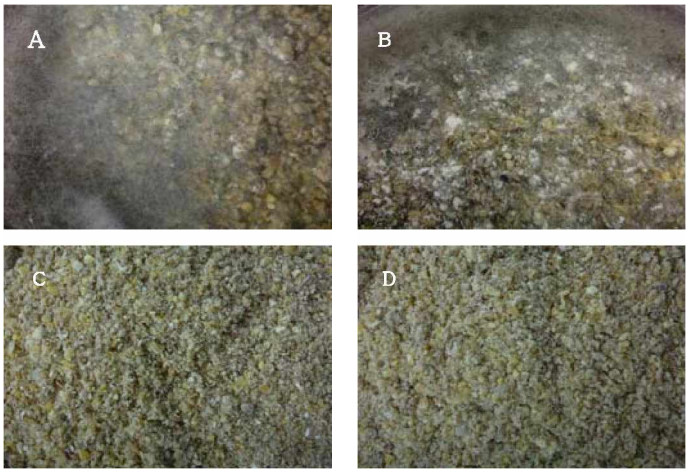

Figure 1. Effect of freeze dried citrus peel (FDCP) on preservation of an antibiotic free diet for swine. Feed samples were incubated for 16 and 21 days at 30±0.5°C, 78-83% humidity. A: day 16 (Control); B: day 21 (Control); C: day 16 (FDCP 22.2 g/kg); D: day 21 (FDCP 22.2 g/kg).

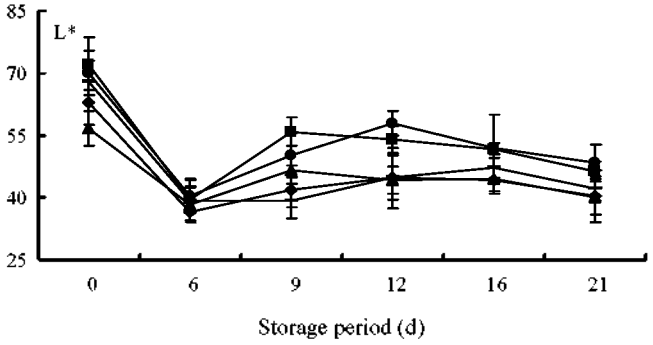

Figure 2. Change in L* colour value of feed samples untreated (Control), treated with an antifungal agent (AA), or treated with freeze dried citrus peel (FDCP), during 21 days of storage. L*, lightness: 0-100 (black-white), ♦: Antibiotic-free feed (control), ▲: 0.05g/kg AA-treated feed, ■ 22.2 g/kg FDCP-treated feed, ●: 11.1 g/kg FDCP-treated feed, —: 5.55 g/kg FDCP-treated feed.

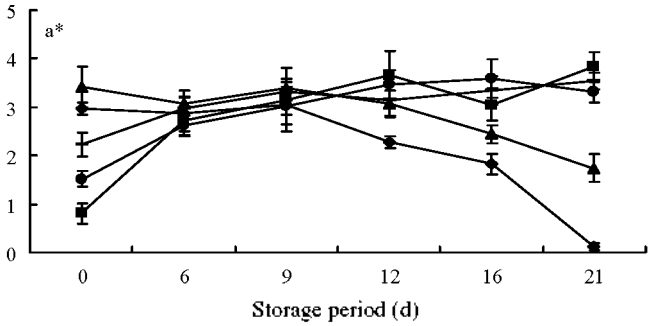

Figure 3. Change in a* colour value of feed samples untreated (Control), treated with an antifungal agent (AA), or treated with freeze dried citrus peel (FDCP), during 21 days of storage. a*, yellowness: +yellow, -blue. ♦: Antibiotic-free feed (control), ▲: 0.05 g/kg AA-treated feed, ■ 22.2 g/kg FDCP-treated feed, ●: 11.1 g/kg FDCP-treated feed, —: 5.55 g/kg FDCP-treated feed.

showed heavy contamination by unidentified microorganisms, but feed samples treated with FDCP (22.2 g/kg) did not show any microbial contamination.

There was no effect of treatment on L* colour values (lightness/darkness), suggesting that this colour value was not influenced by biological contamination in feeds (Figure 2). The a* colour value (redness/greenness) in control samples decreased after 9 days of storage until the end of the experiment (Figure 3); a* colour values for samples treated with AA also declined after 12 days; a* colour values for samples treated with FDCP did not change between days 6 and 21. The b* colour value

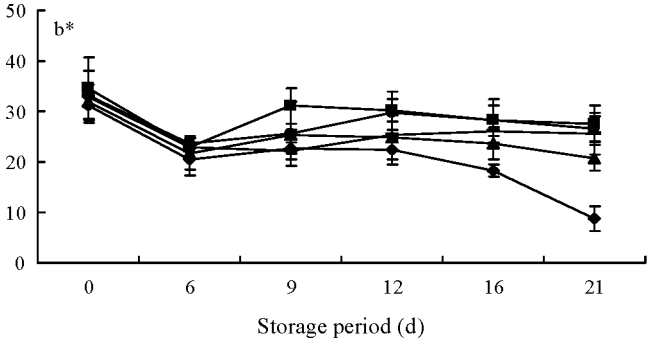

Figure 4. Change in b* colour value of feed samples untreated (Control), treated with an antifungal agent (AA), or treated with freeze dried citrus peel (FDCP), during 21 days of storage. b*, redness, +red, -green. ♦: Antibiotic-free feed (control), ▲: 0.05 g/kg AA-treated feed, ■: 22.2 g/kg FDCP-treated feed, ●: 11.1 g/kg FDCP-treated feed, —: 5.55 g/kg FDCP-treated feed.

(yellowness/blueness) of control and AA-treated feed samples decreased from day 12 (Figure 4), but b* colour values for samples treated with FDCP did not change between days 6 and 21.

Aflatoxin was detected at 16 and 21 days of storage in control feed samples and in samples treated with AA (Table 3). However, none of the feed samples treated with FDCP contained detectable concentrations of aflatoxin. Caccioni et al. (1998) and Nam et al. (2006) reported that volatile components of CEO have antimicrobial action. Freeze drying of citrus peel is likely to retain higher proportions of volatile components from CEO than other methods such as chemical or heat drying. Aflatoxins are produced mainly by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Growth of *A. flavus* and *A. parasiticus* and production of aflatoxins in natural substrates are influenced by a number of factors, including type of substrate, fungal species, moisture content of substrate, relative humidity, and ambient temperature (Viquez et al., 1994). The minimum temperature range for *A. parasiticus* growth is 6-8°C, the maximum is 44-46°C, and the optimum is 25-35°C (Diener et al., 1982). *A. flavus* can produce aflatoxins at temperatures of 12-42°C, but the optimum is 28-30°C (Brackett, 1989). The optimum humidity for both *A. flavus* and *A. parasiticus* is approximately 82% (Brackett, 1989). Temperature and humidity in the current study were near optimal for growth of these fungi. Decreasing colour values a* and b* during storage of untreated feed samples might

Table 3. Aflatoxin content of feed samples untreated (Control), treated with an antifungal agent (AA), or treated with freeze dried citrus pulp (FDCP) at 16 and 21 days of storage

Storage (days)	Treatment (g/kg)				
	Control[1]	AA[2] 0.05	FDCP[3] 5.55	FDCP 11.1	FDCP 22.2
16	8 ppb	2 ppb	ND[4]	ND	ND
21	8 ppb	4 ppb	ND	ND	ND

[1] Antibiotic free feed for swine. [2] Antifungal agent treatment. [3] Freeze dried citrus pulp. [4] Not detected.

Table 4. Effects of addition of freeze dried citrus peel (FDCP) or antifungal agent (AA) on pH, DM digestibility and OM digestibility during *in vitro* rumen fermentation

Item	Incubation time (h)					
	3	6	9	12	24	48
pH						
Control	5.70	5.58[a]	5.43	5.45[a]	5.39	5.45
AA[1]	5.47	5.56[a]	5.50	5.44[a]	5.38	5.45
FDCP[2]	5.47	5.47[b]	5.40	5.30[b]	5.31	5.35
SEM	0.084	0.034	0.030	0.048	0.025	0.033
DM digestibility (%)						
Control	22.5[a]	25.6[a]	38.9[a]	41.5[a]	50.1[a]	60.9[a]
AA	16.2[b]	22.7[a]	38.0[a]	42.0[a]	52.9[a]	60.7[a]
FDCP	32.3[c]	39.3[b]	48.0[b]	49.7[b]	57.6[b]	68.9[b]
SEM	2.85	3.16	2.07	1.61	1.53	1.48
OM digestibility (%)						
Control	19.8[a]	22.7[a]	36.7[a]	39.8[a]	48.5[a]	59.9[a]
AA	13.5[b]	20.3[a]	35.8[a]	40.0[a]	51.5[a]	59.8[a]
FDCP	29.9[c]	37.3[b]	46.1[b]	47.9[b]	56.3[b]	68.0[b]
SEM	2.93	3.29	2.14	1.63	1.57	1.49

[a,b] Means with different superscripts in same column are significantly different ($p<0.05$).
[1] 0.05 g/kg antifungal agent. [2] 22.2 g/kg freeze dried citrus peel.

have been induced by several species of *Aspergillus* moulds, which produce aflatoxins, or by other microbial contamination.

***In vitro* ruminal fermentation**

Effects of treating a ruminant feed with FDCP or AA on pH, DMD and OMD during *in vitro* rumen fermentation are shown in Table 4. Rumen pH was not affected by treatment, except that pH was lower for feed treated with FDCP at 6 and 12 h of fermentation. The pH of some components of the volatile fraction of FDCP, such as limonene, octanol, pinene or citric acid, is generally low. This might have caused the lower pH for feeds treated with FDCP, although it is more likely to have been caused by VFA production. Reduced pH during rumen fermentation of feeds supplemented with citrus pulp was observed by Schaibly and Wing (1974).

DMD and OMD were greater for feed treated with FDCP than for control and AA-treated feed from 3 to 48 h of fermentation. It is likely that these differences were due to the soluble solids in FDCP, which would be rapidly digested. Citrus peel contains a high proportion of neutral detergent-soluble carbohydrates (Gradel and Dehority, 1972). Erickson (1968) reported that citrus peel had a sugar content of 7.6 g/kg and total soluble solids 15.7 g/kg. Total soluble solids include carbohydrates, organic acids, protein and fat. Carbohydrates account for 70 to 80% of total soluble solids in the fruit. The major groups of carbohydrates in citrus fruits include monosaccharides (glucose, fructose), oligosaccharides (sucrose) and polysaccharides (cellulose and pectin). DMD and OMD were significantly lower for AA-treated feed than control feed after 3 h of fermentation. This suggests that propionate might have inhibited microbial digestion of feed during the initial stage of fermentation.

VFA and ammonia-N concentrations during rumen fermentation are shown in Table 5. Concentrations of individual and total VFA were higher ($p<0.05$) for the control feed in the initial stages of incubation (3 and 6 h). However, no difference among treatments was observed after 6 h fermentation. There was no effect of treatment on acetate to propionate ratio. Ammonia-N concentration was higher ($p<0.05$) for the control after 9h fermentation. Ammonia-N production seems to be inhibited by supplementing with AA and FDCP, which might be beneficial for rumen fermentation efficiency (Chamberlain et al., 1985).

The feeds used in these tests were chosen as examples of feeds used for swine and ruminants in Asian countries. A swine diet was chosen for the preservation test because its higher cereal content would make it more susceptible to contamination by aflatoxin-producing moulds under warm humid conditions. A ruminant diet was used for the *in vitro* fermentation study because its higher fiber content would promote digestion by ruminal cellulolytic bacteria, which are more susceptible to antimicrobial agents. Feed samples were ground because, although the suppression effects of FDSP on aflatoxin might be different with other physical forms of feeds, microbial contamination occurs more easily in ground feed than other forms. Untreated feed showed signs of microbial contamination after only 9 days of storage in warm humid conditions, which is consistent with observations in practice. Feed containing FDSP, however, showed no sign of microbial contamination, color change or

Table 5. Effect of treatment with freeze dried citrus peel (FDCP) or antifungal agent (AA) on VFA and ammonia-N concentrations during *in vitro* rumen fermentation

Incubation (h)	Control	AA 0.05 g/kg	FDCP 22.2 g/kg	SEM
Acetate (mM)				
3	23.8[a]	20.1[b]	22.3[b]	1.06
6	24.7[a]	23.4[b]	23.0[b]	0.51
9	25.8	22.3	23.4	1.03
12	26.4	22.2	26.9	1.46
24	23.7	27.4	27.6	1.26
48	25.8[b]	29.2[ab]	31.0[a]	1.53
Propionate (mM)				
3	7.6[a]	6.3[b]	7.1	0.40
6	7.9[a]	7.8[b]	7.5[ab]	0.14
9	8.1[b]	8.4[b]	8.3	0.08
12	9.8	8.5	9.9	0.46
24	10.8	10.9	11.9	0.35
48	9.7[b]	11.9[a]	12.7[a]	0.90
Butyrate (mM)				
3	3.6[a]	3.0[b]	3.4[ab]	0.17
6	4.0[a]	4.0[a]	3.7[b]	0.11
9	4.5[a]	4.3[b]	4.2[b]	0.10
12	4.7	4.4	4.9	0.16
24	5.3	5.3	5.6	0.11
48	5.6	5.8	6.1	0.13
Total VFA (mM)				
3	36.4[a]	30.6[b]	33.6[ab]	1.68
6	35.2	36.7	35.6	0.44
9	40.0	35.6	37.6	1.26
12	42.4	37.0	43.6	2.03
24	40.6	46.3	45.4	1.78
48	43.7[b]	50.2[a]	53.4[a]	2.84
Acetate-propionate ratio (mol/mol)				
3	3.1	3.2	3.1	0.04
6	3.1	2.9	3.0	0.07
9	2.1	2.8	2.8	0.25
12	2.8	2.6	2.7	0.04
24	2.4	2.5	2.3	0.06
48	2.7[a]	2.4[b]	2.4[b]	0.07
NH_3-N (mg/L)				
3	7.2	5.9	6.4	0.38
6	9.7	8.8	8.9	0.26
9	14.2[a]	12.9[b]	12.3[b]	0.55
12	16.7[a]	14.5[b]	14.7[b]	0.72
24	24.2	22.9	23.8	0.36
48	36.8[a]	30.6[ab]	22.7[b]	4.06

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

aflatoxin after 21 days of storage. It is not known how long the preservative effects of FDSP might last beyond 21 days, but even the doubling of storage life found in this study would be of considerable benefit in animal feeding. The lack of detrimental effects on rumen fermentation characteristics suggests that FDSP can be used to preserve ruminant diets as well as swine diets.

CONCLUSIONS

This study shows that FDCP could be added to animal feeds to improve preservation by reducing microbial contamination. The first experiment demonstrated that, in addition to enhancing storage characteristics, FDCP would maintain feed safety by reducing the risk of aflatoxin contamination. The second experiment demonstrated that the antimicrobial properties of FDCP are not detrimental to rumen fermentation at an inclusion level of 22.2 g/kg. These results suggest that FDCP could have beneficial effects on preservation of both swine and ruminant feeds, but further research is needed to investigate possible effects on palatability and animal performance.

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