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Effect of Feeding Dried Orange Pulp to Lactating Dairy Cows on Nutrients Digestibility, Blood Constituents, Plasma Antioxidant Biomarker, and Pathogenic Fecal Bacteria

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ABSTRACT

Experiments were conducted to study the effect of using dried orange pulp (DOP), as an alternative energy source in the ration of Holstein dairy cows, on nutrient digestibility, blood constituents, level of antioxidants in plasma, and presence and count of fecal pathogenic bacteria *e.g. E. coli* and *Salmonella* spp. Forty lactating Holstein cows, weighing 550±50 kg, with an average of 20 kg daily milk yield, were randomly divided into four groups (ten in each group). Animals were fed rations containing DOP at 0, 25, 50 and 75% substitution of yellow corn grains (R1, R2, R3 and R4, respectively). Digestion trail was conducted in the last week of the experiment using Acid Insoluble Ash method. Feces samples were taken and analyzed in the same day for *Salmonella* spp. and *E. coli* and blood samples for analysis were taken from the subcutaneous abdominal vein. The experimental rations were almost identical in chemical composition. Results of nutrient digestibility and nutritive value, as total digestible nutrients (TDN) and digestible crude protein, showed that there were insignificant ($P>0.05$) differences between cows fed R2, R3 and R4 compared with those fed R1 (control), except digestibility of nitrogen free extract which was significantly ($P\leq0.05$) decreased by feeding experimental rations by increasing replacement level of DOP to more than 25% (R2). Blood constituents of experimental cows on all rations were within normal range for platelets, red blood cells (RBCs), hematocrit, hemoglobin (Hb, g/dl), alanine transaminase (ALT), aspartate transaminase (AST), total protein (g/dl), albumin (g/dl), creatinine (mg/dl) and urea. However, white blood cells (WBCs), globulin and total lipids were significantly ($P\leq0.05$) decreased by increasing the replacement level of DOP. Total lipids values decreased by increasing the replacement levels of DOP more than 25% (R2) and total antioxidant capacity (TAC) in blood plasma, regarded as a biomarker of oxidative stress in cows, was insignificantly ($P>0.05$) affected by feeding experimental rations and ranged from 0.420 to 0.433 mm/l. *Salmonella* spp. was not detected and *E. coli* was insignificantly ($P>0.05$) affected by feeding DOP rations. It could be concluded that DOP can be used as an alternative source of energy in Holstein dairy cow's rations, replacing up to 75% of yellow corn grains without negatively effecting nutrient digestibility, nutritive values, as TDN or digestible CP, and without showing any harmful effect on animal health. More studies are needed to examine the effect of citrus by-products on presence of fecal *E. coli* and *Salmonella* species, and on antioxidant biomarkers.

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Authors' Contribution
SMA presented the idea and wrote the article. TME and AEMM statistically analysed the data and revised the article. MHB performed the experiments and analysed the data.

Key words
Dairy cattle, Dried orange pulp, Corn grains, Digestibility, Blood constituents, Plasma antioxidant, Fecal pathogenic bacteria.

INTRODUCTION

Corn grains represent a high percentage of concentrates for ruminants, causing an increase in prices of concentrate feeds. This represents a challenge to animal nutritionists to search for alternative feedstuffs, or agro-industrial by-products that may replace corn grains as a cheaper source of energy in animal rations.

Dried citrus pulp (DCP) is widely used in feeding dairy cows. The dried citrus pulp is a by-product produced after extraction of juice from citrus fruits and drying of the residues. It is classified as an energy concentrate by-product feed. The DCP includes a mixture of peel, inside portions, and culled fruits of the citrus family (*e.g.,* orange, lemons, and grapefruit, *etc.*) that has been dried to produce a coarse, flakey product (Harris, 1991). Source of fruit and method of processing may be considered the main factors that affect the nutrient content of DCP (Ammerman and Henry, 1991).

For sustained and reliable use of non-traditional feedstuffs in animal feed systems, the quantities produced annually and also availability of those feedstuffs must be known. Most of the citrus fruits grown worldwide consist of oranges of various verieties (Hutton, 1987). In 2013/2014, annual world production of oranges was estimated at 50.7 million metric tons (USDA, 2014), which was 1.1 million metric tons higher than its production in 2012/2013 (USDA, 2013). Egypt ranked as the sixth country in global fresh orange production in 2012/2013. Egypt annual production was estimated at 2.450 million tons (USDA,

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2013), and increased in 2013/2014 to 2.570 million tons (USDA, 2014). In Egypt, there are about 85000 tons of fresh orange used for processing juice (USDA, 2013) and produce approximately 51000 ton of fresh citrus pulp (FCP). This massive by-product is not yet extensively used in animal feedstuffs in Egypt and contributes to the environmental pollution. However, drying FCP would be more useful and easier to store and transported to livestock farms. It is estimated that more than 7000 ton of DCP can be produced annually if all FCP is properly dried.

DCP was used at different percentages of substitution with grains (corn, barley, and wheat), dried or pelleted beet pulp, as a part of concentrate feed mixture and sometimes DCP was used as feed supplement to investigate its effect as antioxidant (Castillo *et al*., 2003; Ahooei *et al*., 2011; Allam *et al*., 2011; Santos *et al*., 2014). Effects of inclusion DCP for dairy cow rations on total antioxidant capacity (TAC) in blood plasma as a type of biomarker of oxidative stress and pathogenic bacteria have not been documented.

The objectives of the study were to evaluate the effect of partial substitution of yellow corn grains by dried orange pulp (DOP) as a type of citrus by-products in ration of Holstein lactating cows, on nutrient digestibility, blood constituents, TAC, *Salmonella* and *E. coli* presence in feces.

MATERIALS AND METHODS

Experimental cows, rations and feeding procedures

Forty Holstein lactating cows weighing 550±50 kg, with average of 20 kg daily milk yield, were randomly distributed into four similar groups. Cows were open housed, and were fed four rations (R1, R2, R3 and R4) with concentrates: roughage ratio of 65:35 on DM basis.

Fresh orange juice by-product containing peel, pulp and seeds were collected from plant of food industry and shipped for drying and produce DOP. The control group (R1) received concentrate feed mixtures (CFM) without DOP. Cows in groups 2, 3 and 4 were fed CFM contained respectively 25%, 50% and 75% of DOP as partial substitute with yellow corn grains, respectively (Table I). Corn silage and clover (*Trifolium alexandrinum*) were used as roughages in balancing the experimental rations.

The concentrates and corn silage were offered together 3 times per day at 5.00am, 1.00pm and 9.00pm after milking times, and fresh clover (*T. alexandrinum*) was offered twice daily. Rations were weighed for each group (group feeding). The quantities of daily feed/day/cow were 16 kg CFM + 20 kg corn silage + 10 kg clover. Free drinking water was available all the time. The offered feeds were assessed to cover the nutrient requirements for each dairy cow according to NRC (2001).

Table I.- Formulation of the experimental concentrate feed mixtures.

Ingredients (%)	Concentrate feed mixtures (CFM)			
	R1	**R2**	**R3**	**R4**
Yellow corn	40	30	20	10
Dried orange pulp (DOP)	0	10	20	30
Wheat bran	15	15	15	15
Undecorticated cottonseed meal	15	15	15	15
Lentils	15	15	15	15
Rice bran	5	5	5	5
Soybean meal	5	5	5	5
Limestone	2	2	2	2
Sodium chloride	1	1	1	1
Protected fat	1	1	1	1
Vit. & Min. Mix.*	0.5	0.5	0.5	0.5
Sodium bicarbonate	0.5	0.5	0.5	0.5
Total (%)	100	100	100	100

*Each 3 kg vitamins and minerals mixture contains: Vit. A, 4800000 IU; Vit. D3, 1000000 IU; Vit. E, 28000 mg; zinc, 100000 mg; manganese, 80000 mg; iron, 75000 mg; copper, 30000 mg; iodine, 750 mg; cobalt, 200 mg; silinum, 300 mg; calcium bicarbonate, up to 3 kg. R1, control; R2, 25% DOP; R3, 50% DOP; R4, 75% DOP.

Table II.- Culture medium, counting method and growth conditions were used for detection of *Escherichia coli* and *Salmonella* spp.

Microbial group	Medium	Counting method	Incubation conditions
Escherichia coli	EC broth	MPN	44.5 °C/ 24- 48h.
	L- eosin methylene blue agar	-------	37 °C/ 24h.
Detection of *Salmonella* spp.	Buffered peptone water for the pre-enrichment step.	-------	37 °C/ 24h.
	Selenite cystine broth and Rappaport-Vassiliadis medium for the enrichment step.		37 °C/ 24h.for inoculated Selenite cystine broth and 42 °C/ 24h for inoculated Rappaport-Vassiliadis medium.
	Xylose lacose deoxycholate agar and brilliant green agar as selective medium for isolation of *Salmonella* spp.		37 °C/ 24-48h.

MPN, most probable number.

Sampling and analysis

Samples of feed ingredients, concentrates and feces were analyzed for dry matter (DM), organic matter (OM), ether extract (EE), crude protein (CP) and crude fiber (CF) according to the methods of AOAC (2000). Nitrogen free extract (NFE) was calculated by difference. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed by Ankom200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA) according to van Soest *et al*. (1991). Cellulose and hemicelluloses percentages were calculated by the difference. Gross energy (GE) were calculated according to Edwards and Allan (2004) equations: GE (MJ/kg DM) = 23.6 * CP (g) + 39.5 * EE (g) + 17.2 * carbohydrates (g).

Fecal samples were individually taken from three animals in each group at the end of milk production trial and analyzed on the same day for *Salmonella* spp. according to ISO 6579 (2002) and *E. coli* according to FDA (2002). The culture medium, counting method and growth conditions used for each microbial group are showed in Table II.

Blood samples were collected from three animals in each group at the end of the digestion trial. Blood was withdrawn from the subcutaneous abdominal vein in heparinized tubes at 4 h after morning feeding. Blood hemoglobin (Hb, g/dl) was determined by colorimetric method (Spectrophotometer Jenway 6300 U.K) according to Wintrobe (1956), hematocrit (Ht%), red blood cells (RBCs), white blood cells (WBCs) and platelets determined by Hemocytometer according to Pushkar and Bhatta (2013).

Blood plasma was obtained by spinning the blood at 5000 rpm for 15 min in a clean dried glass vial, and stored at -20°C to determine blood plasma constituents. Blood plasma parameters were analyzed calorimetrically using Jenway 6300 Spectrophotometer U.K. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentration (RFU/ml) were measured according to Reitman and Frankel (1957), albumin (g/dl) as described by Doumas *et al*. (1971), creatinine (mg/dl) according to Bartels (1972), total protein (g/dl) as described by Cannon (1974), total lipids (mg/dl) as described by Eisemann *et al*. (1986) and urea (mg/dl) as described by Fawcett and Scott (1960). Total antioxidant capacity (TAC) was done by using Stat lab szsl60 – Spectrum device according to Koracevic*et al*. (2001).

Digestion trial

The digestion trials were conducted at the last week of the experiment according to van Keulen and Young (1977) using acid insoluble ash (AIA) method. Fecal samples were collected from the rectum of 3 animals in each group, at 10.00 am and 4.00 pm for three successive days, and mixed (six samples for each animal) then stored under deep freezing for later analysis.

Statistical analysis

The obtained data were statistically analyzed using one-way analysis of variance according to XLSTAT (2013) using the following model:

$$Y_{ij}=\mu+T_i+e_{ij}$$

Where, Y_{ij} is observation, μ is general mean of treatments, T_i is effect of treatment and e_{ij} is experimental error.

Differences among means were compared by Duncan's multiple range (Duncan, 1955).

RESULTS AND DISCUSSION

Chemical composition of feed ingredients, CFM and experimental rations

Dry matter composition and cell wall constituents (%) of yellow corn, dried orange pulp, roughages feedstuffs and experimental CFM are shown in Table III. Results indicated that DM %, CP % contents and GE (MJ/kg DM) of DOP and yellow corn grains are approximately equal. The values of OM, NFE, and hemi-cellulose of DOP were found to be lower than that of yellow corn grains. In contrast, values of CF, EE, NDF, ADF, ADL and cellulose were found to be higher than that of yellow corn grains.

The DM and CP for DOP are in agreement with those reported by Ensminger and Olentine (1978) and NRC (1988). The value of GE for DOP is in agreement with those reported by Bampidis and Robinson (2006). The OM and EE content of DOP are near to that found by NRC (2001) and Allam *et al*. (2011). The CF results are in an agreement with these reported by Belibasakis and Tsirgogianni (1996). The NFE value for DOP was to that obtained by Hutton (1987) and Caparra *et al*. (2007). This result for NDF in DOP is in agreement with that of Allam *et al*. (2011) and close to these reported by Hutton (1987) and Belibasakis and Tsirgogianni (1996). Acid detergent fiber (ADF) for DOP was 4.86 times higher than ADF in yellow corn grains (23.80% *vs*. 4.90%). While, the ADF of DOP in the present study is higher than those reported by NRC (1988, 2001), Bampidis and Robinson (2006) and Allam *et al*. (2011) who recorded ADF content of DOP ranging from 16 to 22.2%. Results of ADL from the present study are in agreement with Allam *et al*. (2011).

The concentrate mixtures (Table III) as well as the experimental ration (Table IV) were isocaloric and isonitrogenous. Roughage to concentrate ratio was on average 34.8: 65.2 % in experimental rations on DM basis.

Table III.- Chemical composition (on DM basis) and cell wall constituents (%) of yellow corn, dried orange pulp, roughages feedstuffs and experimental concentrate feed mixtures (CFM).

Items	Feed ingredients				Concentrate feed mixtures (CFM)			
	Yellow corn	DOP	Corn silage	Egyptian clover	CFM1 (Control)	CFM2 (25% DOP)	CFM3 (50% DOP)	CFM4 (75% DOP)
Chemical composition, % (on DM basis)								
DM	90.41	89.66	30.65	16.72	91.49	91.42	91.34	91.27
OM[1]	97.44	92.51	90.56	84.42	90.39	89.90	89.40	88.91
CP	8.07	8.48	8.76	18.63	15.41	15.45	15.49	15.53
CF	3.24	13.85	28.54	30.38	7.53	8.59	9.65	10.71
EE	1.84	4.66	1.23	1.49	4.86	5.14	5.42	5.71
ASH	2.56	7.49	9.44	15.58	9.61	10.10	10.60	11.09
NFE[2]	84.29	65.51	52.04	33.92	62.59	60.71	58.84	56.96
Cell wall constituents (%)								
NDF	23.40	26.36	68.71	53.06	31.19	31.49	31.78	32.08
ADF	4.90	23.80	41.14	39.25	12.20	14.09	15.98	17.87
ADL	1.34	2.20	9.40	10.10	4.08	4.17	4.25	4.34
Hemicelluloses[3]	18.50	2.55	27.57	13.81	18.99	17.40	15.80	14.21
Cellulose[4]	3.56	21.61	31.74	29.15	8.12	9.92	11.73	13.53
GE (MJ/kgDM)[5]	17.69	17.49	16.41	16.04	17.62	17.60	17.58	17.56

DM, dry matter; OM, organic matter; CP, crude protein; CF, crude fiber; EE, ether extract; NFE, nitrogen free extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; GE, growth energy. [1,2,3,4,5] calculated.

Table IV.- Formulation of the experimental rations and its DM composition.

Items	Experimental rations			
	R1	R2	R3	R4
Composition (%)				
CFM	65.23	65.21	65.20	65.18
Roughage	34.77	34.79	34.80	34.82
Corn silage	27.32	27.33	27.35	27.36
Egyptian clover	7.45	7.45	7.46	7.46
Chemical composition				
DM[1]	91.95	91.90	91.85	91.80
OM	89.99	89.67	89.35	89.03
CP	13.83	13.86	13.88	13.91
EE	3.62	3.80	3.98	4.17
CF	14.97	15.67	16.36	17.06
NFE[2]	57.57	56.34	55.12	53.89
Ash	10.01	10.33	10.65	10.97
Cell wall constituents				
NDF	42.04	43.27	43.47	43.67
ADF	22.12	23.36	24.60	25.83
ADL	5.98	6.04	6.10	6.15
Hemicelluloses[3]	19.92	19.91	18.87	17.84
Cellulose[4]	16.14	17.32	18.50	19.68
GE (MJ/kg DM)[5]	17.17	17.16	17.14	17.13

R1, control; R2, 25% DOP; R3, 50% DOP; R4, 75% DOP. Hemicellulose= NDF – ADF; Cellulose = ADF- ADL; [1,2,3,4,5] calculated. For abbreviations, see Table III.

Effect of partial substitution of yellow corn by DOP

Nutrient digestibility and nutritive value of the experimental rations

Partial substitution of yellow corn grains by different levels of DOP (R2, R3, and GR4) did not affect digestibility of all nutrient except NFE (Table V) compared to control group (R1). These results are in agreement with those of Gholizadeh and Naserian (2010), Ahooei *et al*. (2011), Allam *et al*. (2011) and Shdaifat *et al*. (2013).

The ether extract digestibility (EED) coefficient values increased with increasing level of replacement of yellow corn grains by DOP in the rations, but the differences among treatments were not statistically significant. The reasons of increasing values of EED may be due to increased feed intake of EE by 0.18, 0.36 and 0.55% in R2, R3 and R4, respectively (EE intake were increased by 28.8, 57.6 and 88 g/cow/d in R2, R3 and R4, respectively compared to control). Lipids from DCP contain mainly unsaturated fatty acids, which are known to be more digestible than saturated fatty acids. So, the additional amount of digestible EE from DCP may contribute to increase of digestion EE. Also, antioxidants present in DCP could have contributed to increase digestion coefficient of EE (Lima *et al*., 2014). The experimental rations were isocaloric and isonitrogenous. The nutritive values ranged from 64.16 to 66.89% for TDN and ranged from 9.15 to 9.86% for digestible CP.

Table V.- Effect of partial substitution of yellow corn by dried orange pulp in dairy cow ration on nutrients digestibility and nutritive value of the experimental rations.

Items	Experimental groups				SEM
	R1	R2	R3	R4	
Digestion coefficient (%)					
DM	67.13	68.66	66.03	63.79	0.96
OM	69.08	70.18	67.52	66.96	0.84
CP	66.14	67.87	71.05	67.09	1.21
CF	58.62	55.21	60.00	58.26	1.27
EE	83.55	82.85	87.38	89.25	1.34
NFE	71.59[ab]	74.05[a]	67.41[b]	67.95[ab]	1.12
NDF	53.96	55.94	54.11	55.05	1.06
ADF	37.77	41.44	38.60	44.20	1.86
Hemicelluloses	71.94	72.94	74.34	70.77	1.72
Cellulose	55.25	57.26	56.39	56.77	1.30
Nutritive values (% DM basis)					
TDN	65.88	66.89	64.88	64.16	0.74
Digestible CP	9.15	9.41	9.86	9.33	0.17

[a,b,] means of treatments within the same row with different superscript letters are significantly different ($P \leq 0.05$). DOP, dried orange pulp. For abbreviations, see Table III. R1, control; R2, 25% DOP; R3, 50% DOP; R4, 75% DOP.

In dairy cow rations on blood parameters

Increasing level of DOP had no significant effect on platelet count, RBCs, Ht (%), Hb, ALT, AST, Total Protein, albumin, creatinine and urea as shown in Table VI. These results are in agreement with Belibasakis and Tsirgogianni (1996), Allam *et al*. (2011). In contrast, the values of WBCs, globulin and total lipids ($P \leq 0.05$) decreased by increasing the replacement levels of DOP in rations. The results of Total lipids were not in agreement with reported values by Belibasakis and Tsirgogianni (1996), Ahooei *et al*. (2011), Allam *et al*. (2011) and Santos *et al*. (2014).

The value of total antioxidant capacity (TAC) as a biomarker of oxidative stress was gradually increased by increasing level of DOP in experimental rations (ranged from 0.420 to 0.433 mm/l); however, the differences among groups were not significant. More studies are needed to examine the effect of citrus by-products on antioxidant biomarkers especially that TAC in plasma of dairy cow, which is an effective parameter that provides valuable information about the redox conditions of plasma, both dynamically and biologically (Castillo *et al*., 2006). Also, TAC values are a complementary and necessary tool of great value in assessing the metabolic status and monitor health state of dairy cows (Castillo *et al*., 2003). Moreover, the future nutritional recommendations for dairy animals will not be based only on energy and protein requirements but may also based on providing antioxidant needs (Castillo *et al*., 2003).

The partial substitution of yellow corn grains by dried orange pulp in dairy cow ration did not negatively affect animal health and the values of blood parameters of experimental cows, and all values were within normal range.

Table VI.- Effect of partial substitution of yellow corn by dried orange pulp in dairy cow rations on blood analysis.

Items	Experimental groups				SEM
	R1 (Control)	R2 (25% DOP)	R3 (50% DOP)	R4 (75% DOP)	
Platelets (×1000)	392.333	364.333	331.333	301.667	33.528
WBCs (×1000)	12.500[a]	9.733[ab]	9.033[ab]	8.000[b]	1.148
RBCs (×1000000)	6.263	6.02	5.75	5.52	0.278
HT (%)	30.733	30.6	32.233	32.3	2.343
Hb (g/dl)	9.3	9.267	9.767	9.767	0.699
ALT (IU/ml)	11.963	12.44	12.07	12.32	0.37
AST (IU/ml)	16.01	22.473	21.933	21.453	4.551
Total Protein (g/dl)	7.737	7.363	5.873	5.98	0.681
Albumin (g/dl)	3.19	3.823	3.273	3.117	0.235
Globulin (g/dl)	4.547[a]	3.540[ab]	2.600[b]	2.863[ab]	0.559
Total lipids (mg/dl)	637.703[a]	624.590[a]	300.547[b]	395.630[b]	62.504
Creatinine (mg/dl)	0.927	1.15	0.877	0.937	0.095
Urea (mg/dl)	9.383	9.81	15.87	17.24	4.048
Total antioxidant capacity (mm/l)	0.420	0.423	0.430	0.433	0.028

DOP, dried orange pulp; WBCs, white blood cells; RBCs, red blood cells; Ht, hematocrit; Hb, hemoglobin; ALT, plasma alanine transaminase; AST, aspartate transaminase. [a, b,] means of treatments within the same row with different superscript letters are significantly different ($P \leq 0.05$).

On E. coli *and* Salmonella *spp. in feces*

Data in Table VII concerning presence of fecal pathogenic bacteria showed that feces of experimental cows fed experimental rations did not contain *Salmonella* spp. Also, Callaway *et al*. (2011) studied the effects of feeding up to 20% DOP to sheep on fecal *Salmonella* content, but *Salmonella* was not detected and this because sheep did not consume more than 10% DOP diet, likely due to palatability concerns. However, feeding 10% DOP in the ration reduced rune all, cecal and rectal populations of *Salmonella typhimurium* approximately 1, 2 and 0.5 log^{10} CFU (P < 0.08, < 0.06, < 0.10), respectively (Callaway *et al*., 2011).

Data in Table VII showed that cows fed experimental rations did not have significant effect in reducing fecal count of *E. coli* bacteria. The result was surprising because all groups have the same number of *E. coli.* These results are not in agreement with those found by Nam *et al*. (2006), Kirbaşlar *et al*. (2009), Callaway *et al*. (2011), Allam *et al*. (2011), Okunowo *et al*. (2013) and Lee *et al*. (2014) who reported that citrus essential oils reduced *E. coli*.

Table VII.- Effect of partial substitution of yellow corn by dried orange pulp in dairy cow rations on *E. coli* and *Salmonella* spp. in feces of dairy cow.

Experimental groups	*E. coli* (MPN/g)	*Salmonella* spp.
R1 (Control)	1.4×10^3	Not detected
R2 (25% DOP)	1.4×10^3	Not detected
R3 (50% DOP)	1.4×10^3	Not detected
R4 (75% DOP)	1.4×10^3	Not detected

MPN, most probable number; DOP, dried orange pulp.

CONCLUSION

It may be concluded that DOP can replace up to 75% of yellow corn grains in Holstein dairy cow's rations without negatively effecting nutrient digestibility, nutritive values, and blood parameters. Further studies are needed to study the effect of feeding DOP to lactating cows on presence and count of fecal *Salmonella* and *E. coli* in feces, and level of antioxidant biomarkers in milk and blood.

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Statement of conflict of interest

The authors declare no conflict of interest.

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