

Effect of oregano oil dietary supplementation on production parameters, height of intestinal villi and the antioxidant capacity in the breast of broiler

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ABSTRACT. Oregano additives could contain growth stimulating compounds for poultry, however, a great variation attributed to their main components is observed. The aim of the present study was to investigate the effect of oregano oil dietary supplementation on the productive variables, the height of the intestinal villi and the accumulation of antioxidant compounds in the breast of broilers. In total, 480 broilers were reared from 1 to 42 d of age and since the first day of life were allocated into four treatments, each with four replications of 30 birds. The formulated diet was based on corn and soybean meal and 0, 100, 200 or 400 mg of oregano oil per kg of feed were incorporated in the diets of treatment 1, 2, 3 and 4, respectively. Feed intake, weight gain, feed conversion and mortality rates were weekly recorded. At 21 and 42 days of age the intestinal villi height and antioxidant capacity in the chicken breast were measured. Oregano oil used contained 43.17% and 29.16% of thymol and carvacrol, respectively. Feed intake, weight gain, feed conversion rate and mortality were not affected by oregano oil dietary supplementation. The height of intestinal villi increased with the level of oregano oil supplementation ($P<0.05$). However, the size of the intestinal villi was greater in the duodenum compared to the other parts ($P<0.05$), and jejunum and ileum had the same height. Antioxidant capacity of chicken breast at the 6th week was increased in the group that was dietary supplemented with 400 mg of oregano oil per kg of feed.

Key words: thymol, carvacrol, DPPH, natural additive.

RESUMEN. El aceite de orégano contiene aditivos que pueden estimular el crecimiento de las aves, sin embargo presenta gran variación atribuida a sus principales compuestos. El objetivo del estudio fue determinar el efecto del aceite de orégano en la dieta respecto de las variables productivas, altura de las vellosidades intestinales y acumulación de sus compuestos antioxidantes en la pechuga de pollo. En total, 480 pollos fueron engordados de 1 a 42 días de edad, siendo distribuidos en cuatro tratamientos, cada uno con cuatro réplicas de 30 pollos. La dieta se formuló a base de maíz y pasta de soya, adicionada con 0, 100, 200 o 400 mg de aceite de orégano por kg de alimento. El consumo de alimento, ganancia de peso, conversión alimenticia y mortalidad se registraron semanalmente. A los 21 y 42 días de edad se midieron la altura de las vellosidades intestinales y la acumulación de compuestos antioxidantes en la pechuga de las aves. El aceite de orégano contenía 43,17% y 29,16% de timol y carvacrol, respectivamente. La ingesta de alimento, ganancia de peso, índice de conversión alimenticia y mortalidad no fueron afectados por la adición del aceite de orégano a la dieta. La altura de las vellosidades intestinales incrementó con el nivel de aceite de orégano suplementado en la dieta ($P<0,05$), pero el tamaño de las vellosidades intestinales en duodeno fue mayor ($P<0,05$), yeyuno e ileón presentaron el mismo tamaño. Los componentes antioxidantes en la pechuga de pollo incrementaron con 400 mg de aceite de orégano por kg de alimento.

Palabras clave: timol, carvacrol, DPPH, aditivo natural.

INTRODUCTION

Plants contain natural compounds capable of inducing positive effects on poultry productive parameters (Çabuk *et al* 2006, Arce *et al* 2008, Abdel *et al* 2012). Their addition in diets is a growing trend due to the hazards associated with the use of antibiotics, i.e. resistant bacteria that can be transmitted to humans through chicken meat (Castañón 2007). However, the productive response may vary due to the ingredients of the diet, the amount of active components in the additives and the conditions of the experiment (Marković *et al* 2009, Betancourt *et al* 2012). Oregano is a plant grown for culinary purposes and generally improves

the organoleptic characteristics of the food. Oregano oil is obtained from the leaves of the plant and is used in the industry due to its high antioxidant content. Carvacrol and thymol may represent up to 80% of the oil (Stefanaki *et al* 2016). In poultry these compounds can stimulate the digestive processes and protect the polyunsaturated meat fatty acids by decreasing oxidative process rates (Rocha-Guzmán *et al* 2007, Botsoglou *et al* 2003, Marčincák *et al* 2008).

Natural additives can improve the weight gain of poultry, feed intake and feed conversion. Their effects have also been studied on the digestive organs development, gastric secretions, digestibility of dietary nutrients and the integrity of the digestive cells (Hernández *et al* 2004, Shiva *et al* 2012). The size of the intestinal villi could serve as an indicator of the general state of the digestive tract because these structures are sensitive to the ingredients contained in the diets (Viveros *et al* 1994, Betancourt *et al* 2012). As a result, the size of the intestinal villi could be related with the broiler ability to absorb nutrients and the improvement of growth (Onderci *et al* 2008, Incharoen *et al* 2010). Therefore, the aim of the study was to determine the effect of oregano oil dietary supplementation

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on production variables, height of intestinal villi and the meat antioxidant capacity in broilers.

MATERIAL AND METHODS

POULTRY AND EXPERIMENTAL DIETS

The research was conducted at the poultry farm of the Professional Academic Unit of Veterinary Medicine and Husbandry at the Universidad Autónoma de Nayarit, located in the municipality of Compostela, Nayarit, Mexico. The methods used in the present experiment were approved by the bioethical committee of this educational center. A total of 480 Ross 308 broilers of both sexes were used; they were fed for 42 days a diet based on corn-soybean meal (table 1) balanced according to the NRC recommendations

Table 1. Chemical composition (%) of diet.

Ingredient	Initiation	Grow Finalization
Corn	61.17	62.67
Soybean meal	31.45	29.48
Fish Flour	1.00	1.00
Soybean Oil	3.50	3.60
Calcium Bicarbonate (38%)	1.43	1.52
Dicalcium Phosphate (18/21)	0.78	0.95
Salt	0.30	0.30
Vitamin Premix ¹	0.01	0.01
Vitamin Premix ²	0.02	0.02
DL-Methionine	0.16	0.09
HCL-Lysine	0.18	0.06
Xantophylls ³	0.00	0.30
	100.00	100.00
Calculated analysis		
Mega calories per kilogram of feed	3.05	3.10
Crude protein	20.50	19.50
Calcium	1.00	0.90
Lysine	1.25	1.10
Methionine + cystine	0.94	0.78
Methionine	0.50	0.40
Available phosphorus	0.45	0.35
Threonine	0.80	0.70
Tryptophan	0.23	0.20
Isoleucine	0.88	0.70
Valine	0.95	0.76
Arginine	1.25	1.05
Linoleic acid	1.00	1.00

¹ Quantity in mg per kg feed: vitamin A, 10,000 IU; vitamin D₃, 2,500 IU; vitamin K₃, 2 mg; thiamine, 2 mg; riboflavin, 7 mg; pantothenic acid, 10 mg; pyridoxine, 4 mg; folic acid, 1 mg; Vitamin B12, 0.015 mg; and biotin 0.010 mg (Vipresa®), Tepatitlán de Morelos, México.

² Quantity in mg per kg feed: Se, 0.20; I, 0.30; Cu, 7; Fe, 65; Zn, 75; Mn, 65; y Co, 0.4 (Vipresa®), Tepatitlán de Morelos, México.

³ Quantity in mg per kg feed: 90 ppm *Tagetes erecta* (Florafil-93 Powder, Industrias Vepinsa S.A. de C.V). Los Mochis Sinaloa, México.

(1994). Birds were randomly allocated to four treatments, each with four replications of 30 broilers. The basal diet was supplemented with 0, 100, 200 or 400 mg of oregano oil per kg of feed (T-1, T-2, T-3 and T-4, respectively) at the time of diet preparation both in the initiation and growth-finalization stages. Feed and water were provided *ad libitum* during the 42 days of the experiment; feed intake, weight gain, feed conversion, and mortality rates were weekly recorded.

OREGANO OIL COMPOSITION

The compounds of oregano oil were identified using a gas chromatograph (Hewlett Packard P-6890, California, USA) coupled to a mass spectrometer (Hewlett Packard 7953, California, USA). The injection port temperature was 240 °C; Hewlett Packard capillary column 5ms® (30 m long, 0.25 mm internal diameter and 0.25 µm of film thickness, California, USA) was used. The initial oven temperature was 50 °C for 5 min and then increased 10 °C per minute up to 260 °C; Helium was used as carrier gas. The mass spectrometer was operated in scan (range m/z 30-550) electron ionization mode (70 eV) and flow rate of 1.0 mL/min.

SACRIFICE AND CHICKEN BREAST SAMPLES COLLECTION

At the end of the experiment (42 days) two birds per repetition were sacrificed by cutting the jugular vein section and carotid artery following the recommendations of the Official Mexican Standard NOM-033-ZOO-1995. They were bled for 2 minutes, scalded (60 °C) for 120 seconds and manually plucked. Carcasses were cooled in iced water for one hour (about 0 °C). Chicken breasts were skinned and after visible fat was removed, they were split in half and the right part was packaged in high vacuum to be frozen (-20 °C) until analysis.

HEIGHT OF INTESTINAL VILLI

Samples of the duodenum, jejunum and ileum mid-points of two birds per replicate (eight per treatment) were collected for each treatment at 21 and 42 days of age; as duodenum was considered the part from loop along the pancreas up to bile ducts insertion, as jejunum the midpoint between the bile ducts entrance and Meckel's diverticulum, and as ileum the area 10 cm proximal to the ileocecal junction. Samples were cleaned with distilled water to remove the intestinal contents and fixed in formalin buffered [1 liter of 10% formaldehyde + 4.0 g sodium phosphate monobasic (NaH₂PO₄·H₂O) + 6.5 g dibasic sodium phosphate (Na₂HPO₄)]. Tissue samples were embedded in paraffin for cuts to 6 µm (Microtome, Leica®). Histological sections were stained with hematoxylin and eosin, the height of the intestinal villi was determined measuring from the base of the lamina

propria to the tip of the villi for duodenum, jejunum and ileum. 15 intestinal villi per sample were measured for each intestinal section and for each bird (Image Analyzer Leco Instruments 2001, Version 2.02).

ANTIOXIDANTS EXTRACTION FROM CHICKEN BREAST

For the measurement of the antioxidant compounds accumulated in breast, the technique of Tejerina *et al* (2012) after modifications was used. Six grams of breast were placed in 30 mL of 80% ethanol to grind for 20 s (Blender, Moulinex®) and each sample was allowed to settle in the dark (25 °C) for 24 h. The resulting solution was stirred and filtered to 0.20 µm (Phenex Syringe®).

Indirect measurement of accumulated antioxidants in breast meat was performed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical technique. The DPPH reagent is radical stable in an ethanol solution, has strong violet coloration and its maximum absorbance is obtained at 517 nm. After adding the extract from the chicken breast, the DPPH solution reacts immediately and the color changes to yellow. The antiradical activity based on DPPH· was evaluated with Trolox® (6-hydroxy carboxylic-2, 5, 7, 8-tetrametilcromo, Sigma Aldrich, Toluca, State of Mexico, Mexico) vitamin E analogous molecule.

A solution of DPPH was prepared at a concentration of 7.4 mg in 100 mL of ethanol, stirring for 10 minutes in the dark. 100 µL of meat extraction were added in 500 µL of the DPPH· solution. This solution stirred and allowed to settle in the dark (20 °C) for 30 min, the absorbance of meat was measured with 517 nm. A standard curve was prepared with 500µmol of trolox equivalents per mL.

STATISTICAL ANALYSIS

Feed intake, weight gain, feed conversion, the size of the intestinal villi and the equivalents of trolox accumulated in breast meat were compared with an experimental design completely randomized using the generalized linear procedure (GLM) of SAS (2000). Comparison of statistical means was performed with the Tukey test. Mortality data were transformed using the Arc sine function and presented as a percentage of weekly mortality. The statistical model used was $Y_{ij} = \mu + T_i + \epsilon_{ij}$. Where: Y_{ij} was feed intake, weight gain, feed conversion, size of the intestinal villi and equivalents of trolox in breast meat, μ was the measure using a generalized, μ was the overall mean, T_i was the effect of oregano oil added in the diet and ϵ_{ij} was the random error.

RESULTS

OREGANO OIL COMPOSITION

The identified compounds in oregano oil are presented in table 2 and the chromatogram peaks and retention times are shown in figure 1. The compounds with the highest

Table 2. Oregano oil composition.

Compound	Retention time	Content (%)
P-cymene	6.01	2.90
Eucalyptol	6.13	6.96
γ-Terpinene	6.51	1.80
Linalool	7.07	0.91
Borneol	8.14	0.90
L-4-terpineol	8.30	1.61
α-pinene	8.47	1.31
Rimantadine	8.63	0.57
Thymol methyl ether	9.05	0.95
Thymol	9.83	43.47
Carvacrol	9.98	29.16
Cariophyllene	11.71	5.38
Tetramethyl	12.15	2.96
Acetophenone	12.35	0.40
β-Caryophyllene	13.76	0.75

percentages in oregano oil were: thymol (43.47%), carvacrol (29.16%), eucalyptol (6.96%), caryophyllene (5.38%) and tetramethyl (2.96%).

PRODUCTION VARIABLES

Feed intake, weight gain, feed conversion and mortality rates are presented in table 3. There was no effect of the different levels of oregano oil inclusion in the diet on any of the productive parameters.

HEIGHT OF INTESTINAL VILLI

Table 4 shows the data on the height of intestinal villi in three and six weeks old chickens. In three weeks, duodenum villi of T-4 group was the largest among the experimental groups, and T-2 and T-3 groups had greater values compared to controls ($P<0.05$). In jejunum, T-3 and T-4 groups had higher villi compared to T-1 and T-2 groups ($P<0.05$). Intestinal villi of ileum was higher in T-4 compared to that of the other groups, with a significant difference only between T-4 and T-1 broilers ($P<0.05$). When comparing the height of the intestinal villi in duodenum, jejunum and ileum, duodenum of T-4 group had higher intestinal villi compared to jejunum and ileum ($P<0.05$).

In six weeks, height of the intestinal villi in duodenum was higher in T-4 group, followed by T-3 and finally by T-2 and T-1 groups ($P<0.05$). In jejunum, T-4 had also the highest intestinal villi, followed by T-3, T-2 and the control group ($P<0.05$). In ileum, T-4 had higher villi followed by T-3 and finally T-2 and control group ($P<0.05$). The height of intestinal villi in duodenum was greater compared to jejunum and ileum among all the experimental groups ($P<0.05$).

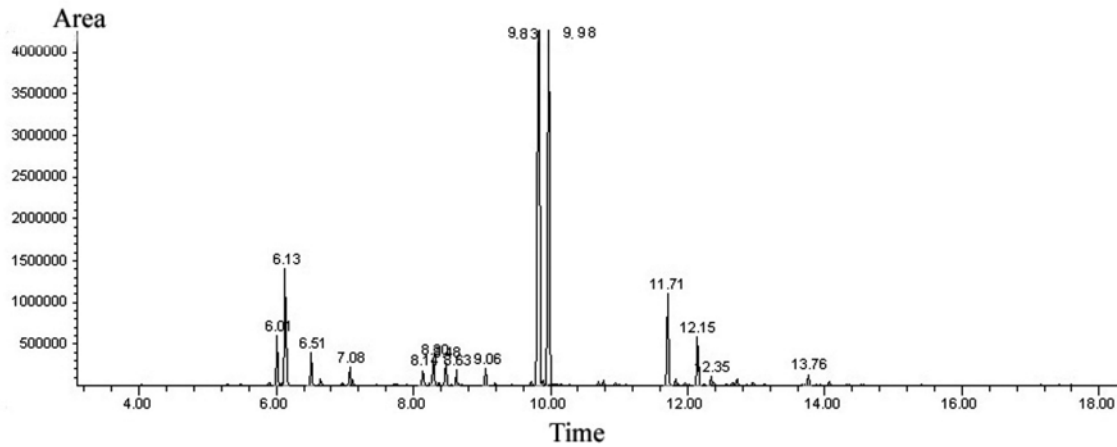


Figure 1. Chromatographic profile of oregano oil.

Table 3. Effect of oregano oil in the production parameters of broilers.

Treatment	Starter			Grower-finisher		
	Week					
	1	2	3	4	5	6
	Weekly feed intake (g)					
T-1	141.20	314.85	485.33	643.48	808.15	1533.55
T-2	139.13	318.11	485.52	650.66	897.59	1502.62
T-3	137.00	306.24	477.45	630.85	806.80	1472.28
T-4	135.03	310.63	467.66	624.15	853.24	1548.00
P > F	0.2710	0.3876	0.3326	0.3696	0.3467	0.3656
SEM	2.04	7.83	16.66	23.2	68.8	53.4
	Weight gain (g) / week					
T-1	94.43	203.60	270.05	423.94	484.94	617.18
T-2	95.53	212.97	262.05	474.13	529.55	640.28
T-3	88.80	207.92	258.01	435.73	458.68	597.36
T-4	87.38	207.61	238.99	437.78	520.40	578.19
P > F	0.1499	0.6383	0.6164	0.6089	0.6436	0.6386
SEM	3.33	9.31	12.03	25.82	28.88	40.63
	Feed conversion rate (kg)					
T-1	1.50	1.55	1.80	1.54	1.65	2.49
T-2	1.46	1.50	1.86	1.38	1.71	2.36
T-3	1.55	1.48	1.87	1.46	1.77	2.52
T-4	1.56	1.51	1.99	1.43	1.65	2.71
P > F	0.2710	0.6383	0.3876	0.3278	0.3766	0.3812
SEM	0.04	0.05	0.10	0.07	0.13	0.1512
	Mortality (%)					
T-1	0	0.83	0.84	0	0	0
T-2	0	0.83	1.68	0	1.73	0
T-3	0	0.83	2.52	0	0	0
T-4	1.66	0.84	1.70	0	0	0
P > F	0.0728	1.000	0.6514	SA	0.0728	SA

T-1: without oregano oil.
 T-2= oregano oil at 100 mg per kg of feed.
 T-3= oregano oil at 200 mg per kg of feed.
 T-4= oregano oil at 400 mg per kg of feed.
 SEM: Standard error of the mean.
 SA: Unanalyzed.

Table 4. Means (µm) intestinal villi height in chickens three and six weeks of age.

Treatment	Duodenum	Jejunum	Ileum	<i>P</i> > <i>F</i> ¹
	Week 3			
T-1	602.29 c	564.34 b	640.76b	0.0964
T-2	795.31 bx	606.49 by	742.50ab x	<0.0001
T-3	864.75 b	747.61 a	764.58ab	0.1810
T-4	1223.79 ax	833.31 ay	843.39 ay	<0.0001
SEM ²	30.22	21.90	24.47	
<i>P</i> > <i>F</i>	<0.0001	<0.0001	0.0428	
	Week 6			
T-1	1369.50 cx	719.69 dy	878.56 cy	<0.0001
T-2	1322.13 cx	1034.85 cy	949.41 cy	<0.0001
T-3	1743.37 bx	1255.86 by	1152.39 by	<0.0001
T-4	2111.31 ax	1492.53 ay	1396.21 ay	<0.0001
SEM	40.91	38.10	32.77	
<i>P</i> > <i>F</i>	<0.0001	<0.0001	<0.0001	

T-1: without oregano oil.

T-2= oregano oil at 100 mg per kg of feed.

T-3= oregano oil at 200 mg per kg of feed.

T-4= oregano oil at 400 mg per kg of feed.

¹*P* > *F* Probability.

²SEM: Standard error of the mean.

^{a-c}Means with different superscript within the same column are statistically different (*P*<0.05).

^{x-z}Means with different superscript within the same row are statistically different (*P*<0.05).

DETERMINATION OF ANTIOXIDANT CAPACITY IN CHICKEN BREAST

Table 5 illustrates the antioxidant capacity in breast samples of broilers. In 3 weeks, oregano oil dietary supplementation (100, 200 or 400 mg) increased antioxidant capacity compared to the control (*P*<0.05). In 6 weeks of age, only chickens of T-4 group had greater antioxidant values compared to the controls (*P*<0.05).

DISCUSSION

OREGANO OIL COMPOSITION

The concentration of oregano oil compounds varies due to the harvest time, cultivation conditions, geographical location, altitude above sea level, climate, genetic variety and plant's phenological age when cut (Abu-Lafi *et al* 2008). The oregano oil, used in the present study, contained similar levels to those reported in European oregano oils (concentration of thymol and carvacrol at the level of 70%), but higher levels than that of Mexican oregano oils (Arana-Sánchez *et al* 2010).

PRODUCTION VARIABLES

There was no effect of oregano oil dietary supplementation on the broiler productive parameters. Botsoglou

Table 5. Antioxidant capacity of chicken breast in 3 and 6 weeks of age (expressed as milliequivalents of Trolox per six grams of meat).

	Week 3	Week 6
T-1	0.694b	0.705 ^b
T-2	0.725a	0.726 ^{ab}
T-3	0.727a	0.727 ^{ab}
T-4	0.727a	0.734 ^a
<i>P</i> > <i>F</i> ¹	0.0019	0.0187
SEM ²	0.002	0.003

T-1: without oregano oil.

T-2= oregano oil at 100 mg per kg of feed.

T-3= oregano oil at 200 mg per kg of feed.

T-4= oregano oil at 400 mg per kg of feed.

¹*P* > *F* Probability

²SEM: Standard error of the mean.

^{a-b}Means with different superscript within the same column are statistically different (*P*<0.05).

et al (2002) and Marčincák *et al* (2008) obtained similar results by adding 50, 100, 200 and 500 mg of oregano oil per kg of feed. The amount of thymol and carvacrol in oregano oil has been linked to weight gain, since these compounds stimulate the secretion of bile, pancreas and digestive enzymes leading to an improved absorption of nutrients in the intestinal lumen (Russo *et al* 1998, Lee *et al* 2004, Wang and Peng 2008). Although, stimulation

of secretion in the digestive system could possibly be an effect of oregano oil, it was not associated with an improvement of the productive parameters in the present study. On the other hand, Nihat *et al* (2005), Betancourt *et al* (2012) and Freitas *et al* (2013) reported improvement in production variables due to the presence of thymol in oregano oil. Similar results were reported with various combinations of Mexican oregano oil (Vázquez *et al* 2015). In addition, the diet ingredients could alter the effect of the components of oregano oil as reported by Uni *et al* (1998) and Langhout *et al* (2000), who mentioned that the digestibility of the ingredients and the environmental conditions could play an important role and influence the effects of added natural additives. For example, the use of a less digestive or contaminated feed could result in a greater effect of natural additives on the parameters of broilers.

HEIGHT OF INTESTINAL VILLI

The intestinal villi height depends on the balance between proliferation, migration and cell apoptosis. The main function of the villi is the absorption of nutrients, an activity which is partially dependent on its size. In addition, villi can be considered as intestinal health indicator (Uni *et al* 1998, Laudadio *et al* 2012). Natural additives improve the epithelium surface structure and increase the enterocytes size (Wang and Peng 2008, Incharoen *et al* 2010).

The highest villi appeared in 42 days old chickens, and the size in duodenum, jejunum and ileum was greater by 16, 22 and 70%, respectively, compared to that reported by Chiang *et al* (2010) and Zavarize *et al* (2012). Other researchers report no morphological changes at doses of 500 mg of oregano oil per kg of feed (Onderci *et al* 2008, Betancourt *et al* 2012, Emami *et al* 2012, Laudadio *et al* 2012, Wu *et al* 2013). Olnood *et al* (2015) mentioned that additives could favor the development of bacteria and improve growth of the anatomical structures. However, the variation of components in oregano oil and diet are factors that can modify these effects. Chiang *et al* (2010) and Sayrafi *et al* (2011) report that the villi height increases with the amount of oregano oil in the feed, an effect that was also observed in the experiment both for 3 and 6 week old chickens.

ANTIOXIDANT CAPACITY OF CHICKEN BREAST

The height of intestinal villi was associated with the accumulation of antioxidant compounds in the breast meat, since villi height influences the absorption of nutrients (Chiang *et al* 2010). Chickens receiving a diet with 400 mg of oregano oil per kg feed had higher absorption and accumulation of oregano compounds and its metabolites in their muscle fibers (Uni *et al* 1998). The amount of compounds accumulated in the breast meat was higher in chickens that received higher levels of oregano oil.

It can be concluded that oregano oil that contained 43% thymol and 29% carvacrol resulted in no effect on broilers' productive parameters at the doses of 100, 200 or 400 mg per kg of feed. However, by increasing the dose of oregano oil, height of the intestinal villi was also increased. The larger size of the villi appeared in duodenum, jejunum and ileum in six weeks old broilers. Finally, antioxidant capacity of breast meat was higher in chickens fed with the oregano supplemented diets compared to the controls.

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