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OREGANO ESSENTIAL OIL AS ANTIMICROBIAL AGENT IN BROILERS DIET

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ABSTRACT: The study was carried out to evaluate the dietary supplementation of oregano essential oil on performance and antimicrobial activity of broilers. A total of 250 day-old male were distributed in a completely randomized design divided into five groups with five replicates, each given a basal diet with avilamycin plus salinomycin (positive control) or a basal diet without added avilamycin and salinomycin (negative control) or basal diet with only added avilamycin or a basal diet supplemented with 0.5g of oregano oil/kg diet, or 1.0g of oregano oil/kg diet. It was observed lower frequency of gram-positive bacteria in the ileum of the negative control group compared to chickens treated with the positive control group or supplemented with oregano oil ($p < 0.01$). *Lactobacillus* spp. had a higher growth in the positive control group or supplemented with 1g of oregano oil/kg diet ($p < 0.05$). However, the frequency of *Lactobacillus* spp. in broilers supplemented with 0.5g of oregano oil/kg diet was lower than those supplemented with 1g of oregano oil/kg diet ($p < 0.05$) as well, as it did not differ from the negative control group ($p > 0.05$). It was observed that the use of 1.0g of oregano oil/kg diet resulted in the lower relative intestine weight ($p < 0.05$) which did not differ significantly from the positive control group ($p > 0.05$). The results showed that the dietary oregano oil exerted growth-promoting effect on broilers at 1.0g of oregano oil/kg diet by the increase in the *Lactobacillus* spp. frequency and the decrease in intestinal weight in broilers.
Key Words: chickens, ileum, microbiota, growth promoter antibiotic, terpenes

ÓLEO ESSENCIAL DE ORÉGANO NA DIETA DE FRANGOS COMO AGENTE ANTIMICROBIANO

RESUMO: O presente estudo objetivou avaliar a adição de óleo essencial de orégano como agente antimicrobiano e sobre o desempenho de frangos de corte. Utilizou-se 250 pintos de corte, machos, da linhagem Cobb, distribuídos em um delineamento inteiramente casualizado com cinco tratamentos e cinco repetições, com 10 aves cada: dieta com antibiótico e anticoccidiano (controle positivo); dieta sem antibiótico e anticoccidiano (controle negativo); dieta contendo apenas antibiótico; dieta com 0,5g de óleo de orégano; dieta com 1,0% de óleo de orégano. Foi observada uma menor frequência de bactérias gram-positivas no íleo de frangos de corte tratados sem promotor de crescimento comparado com os grupos controle positivo ou com óleo de orégano ($p < 0,01$). Dentre as bactérias gram-positivas, os *Lactobacillus* apresentaram uma maior taxa de crescimento no íleo de frangos tratados com promotor de crescimento ou com 1g de óleo de orégano/kg de dieta. Porém, nos animais tratados com 0.5g de óleo de orégano foi verificada uma menor taxa de crescimento de *Lactobacillus* comparado com os tratados com 1g de óleo de orégano ($p < 0,05$), não diferindo do grupo controle negativo ($p > 0,05$). A adição de 1g de óleo de orégano/kg de dieta resultou em um menor peso relativo dos intestinos ($p < 0.05$), que não diferiu do grupo controle positivo ($p > 0.05$). Os resultados mostraram que a adição de 1g de óleo de orégano/kg de dieta exerceu um efeito antimicrobiano verificado por meio da redução do peso relativo dos intestinos e pelo aumento na frequência de *Lactobacillus*.
Palavras-chave: aves; íleo; microbiota; antibiótico promotor de crescimento; terpenos

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INTRODUCTION

In Brazil, at the first quarter of 2011 were slaughtered 8,160 million of pigs and 1,306 billion of chickens (Brasil, 2011). To ensure the productivity and competitiveness of the agribusiness, the use of drugs as growth promoters is a common practice. The infectious diseases that affect the digestive tract of broilers has as one of the main causes economic losses. Faced of this situation, production systems have been based on the control of infectious diseases through the use of growth promoter antibiotics. However, the use of them has been discussed due to the emergence of resistant bacteria. In response to this, for some years it is encouraged the search of alternatives like as essential oils. Plants are a rich source of such compounds with synergistic properties (Gibbons, 2005).

Natural, complex, multi-component essential oils are composed mainly of terpenes such as thymol, cavacrol, citral and others non-terpene components (Edris, 2007). Strong *in vitro* evidence indicates that essential oils can act as antibacterial agents against a wide spectrum of pathogenic bacterial strains including *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenterica*, *Bacillus cereus* and *Staphylococcus aureu*s (Burt, 2004). The essential oils are designated as generally recognized as safe (GRAS) by the United States, FDA (Godwin and Michniak, 1999; Williams and Barry, 1991). Antimicrobial action of carvacrol and thymol, the main components of oil of oregano has been reported by Burt (2004).

According to Hersch-Martinez, Leaños-Miranda and Solórzano-Santos (2005) the oregano essential oil was effective against gram-negative pathogen. Penalver *et al.* (2005) verified that the oregano essential oil showed antimicrobial activity with a minimum inhibitory concentration of equal to or less 1%. It has been demonstrated, *in vitro*, the antimicrobial effect of essential oils but their influence on growth performance of farm animal species has not been sufficiently documented. This study to evaluated the effects of oregano essential oil on performance and antimicrobial activity of broiler chickens.

MATERIAL AND METHODS

A total 250 day-old male Cobb chicks were housed indoors, on twenty five floor pens, and distributed in a completely randomized design divided into five treatment with five replicates (ten broilers/box) given a basal diet with 10mg/kg avilamycin plus 66mg/kg salinomycin (positive control); a basal diet without added avilamycin and salinomycin (negative control); basal diet with only added avilamycin at 10mg/kg (only antibiotic); basal diet supplemented with 0.5g of oregano oil/kg diet and basal diet with 1.0g of oregano oil/kg diet.

The feeding program consisted of a starter diet until 19 d and a finisher diet until 42 d. The birds were fed a start diet containing 22% of crude protein and 2900 kcal of metabolizable energy/kg, and a growing diet with 19% of crude protein, and 3000 kcal of metabolizable energy/kg. The basal start diet had 1.5% of soybean oil and growing diet had 2% of soybean oil. Oregano oil supplementation was obtained by isometrically replacing soybean oil in the basal diet. Feed and water were supplied *ad libitum.*

Commercial samples of oregano (*Origanum vulgare*) of chilean origin were used to obtain the essential oil by hydrodistillation in a modified Clevenger-type apparatus, and their analyses were performed by gas-chromatograph with flame ionization detector (GC/FID) according to Cleff *et al.* (2008).

The feed intake, weight gain and

feed conversion were calculated. The animals were inspected daily and dead birds were removed following registration of date and body weight. When calculating feed conversion (kg diet/kg weight), the body weights of dead animals were also considered.

Five birds per treatment were sacrificed by cervical dislocation; the small intestine was rapidly excised at Meckel's diverticulum to the ileocecal junction; the ileum were removed aseptically, clamped with forceps, and placed in sterile plastic bags on ice. In the laboratory, the narrow open ends of the ileum were cut with sterile scissors. Approximately 100mg of ileal content was collected into an eppendorff tube containing about of 1mL of of sterile reduced sodium thioglycollate broth, and homogenized by mild agitation for three seconds. The samples were submitted to a serial decimal dilution in sterile reduced sodium thioglycollate broth up to 10^{-6}. However only 10^{-6} dilution were considered for bacterial CFU counts with 0.1mL was plated into culture media for the identification of bacteria. All culture media - Mueller-Hinton blood agar, Man Rogosa Sharp (MRS) agar, Azide blood agar, MacConkey agar - were incubated in an anaerobic cabinet (GasPak, BBL) at 37ºC for 48h.

The *Lactobacillus* spp. count was determined presumptively using MRS agar (BBL. Beckton Dickinson, USA). From azide blood agar plates showing pure growth, two typical colonies were subcultured for separation between *Enterococcus* and *Streptococcus* species, by verifying the ability for enzymatically hydrolase a l-pyrrolidonyl-beta-naphthalamide substrate as well as positive for esculin hydrolysis and positive growth with 6.5% (wt/vol) sodium chloride brain heart infusion broth at 45°C. Also, each colony presenting distinct morphology was isolated, stained by Gram and tested for catalasys. Others enterobacteria were enumerated on MacConkey agar after anaerobic incubation at 37°C for 48h. Besides, the relative intestine weight was determined (small intestine and ceca) at 19 and 42 days of age.

The experimental data was subject to an analysis of variance using the System for Statistical and Genetic Analyses, developed by UFV (1997). Significant differences among means were determined by Student Newman-Keul´s (SNK) test at $p \leq 0.05$ throughout these studies. Microbiology parameters were compared using the Fisher test at a probability of 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Investigations concerning to the growth performance and antimicrobial activity of the essential oils of the plant have been conducted in the search for new compounds to replace synthetic ones. In our results, the extraction yield of the oregano essential oil was determined to be 1.20 wt% ± 0.18 wt%, achieved after about 3h of extraction. The chromatogram of oregano essential oil shows that 4-terpinenol, γ-terpinene and thymol are the major components, followed by α-terpinene, p-cymene and α-terpineol, which means a chemical profile very similar to that found by Rodrigues (2002).

It was shown in this study that *O. vulgare* L. had a high percentage of thymol compared to carvacrol. With respect to the terpene compounds, several researches have indicated higher inhibition of microorganisms by thymol followed by carvacrol (Lattaoui and Tantaoui-Elaraki, 1994).

There was no effect of treatments on body weight gain ($p > 0.05$). Similar results were observed by Rizzo *et al.* (2010) that reported no differences in final body weight among chickens fed oregano. According Traesel *et al.* (2011), essential oil of oregano, in highest dose, exerts less stimulating

humoral immune system of broilers, as with the supplementation of growth-promoting antibiotics.

It was observed that the positive control group had the highest feed intake ($p \leq 0.05$) whereas that the broilers fed with 1 g of oregano oil/kg diet had better fed conversion at 1-19 days of age without to influence negatively feed consumption (Table 1).

Table 1. Growth performance of broilers chickens

Diet	1-19 days	20-42 days	1-42 days
	Body weight gain (g/bird)		
Positive control [1]	412.21	1451.21	1870.43
Negative control [2]	376.45	1498.06	1880.36
Only antibiotic [3]	392.18	1551.38	1948.02
0.5g of oregano oil/kg	370.05	1412.01	1787.66
1.0g of oregano oil/kg	429.42	1485.95	1925.75
	Feed intake (g/bird)		
Positive control [1]	740.16A	2962.05	3702.22
Negative control [2]	665.48AB	2932.30	3597.78
Only antibiotic [3]	693.12AB	3084.17	3747.29
0.5g of oregano oil/kg	627.08B	2871.37	3498.45
1.0g of oregano oil/kg	697.36AB	3090.00	3787.36
	Feed conversion (feed:gain ratio)		
Positive control [1]	1.79 A	2.04	1.98
Negative control [2]	1.76 A	1.95	1.91
Only antibiotic [3]	1.77 A	1.98	1.92
0.5g of oregano oil/kg	1.69 AB	2.03	1.95
1.0g of oregano oil/kg	1.62B	2.08	1.96

[A, B] Averages values within the same column with no common superscript differ significantly by the SNK test ($p < 0.05$).
[1] diet with antibiotic and anticoccidial - 10mg/kg avilamycin plus 66mg/kg salinomycin
[2] diet without antibiotic and anticoccidial
[3] 10mg/kg avilamycin

It was focused on the ileal microbiota of broilers because previous studies showed that this population of microorganisms is similiar to the jejunum (Van Der Wielen *et al.*, 2002). According to Lu *et al.* (2003) in the ileum of broilers chickens, *Lactobacillus* species accounted for 67% of the total, with the majority of the rest being related to *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%), totaling 91% of gram-positive bacteria. Our results of microbiology assay showed that the broilers chickens fed with growth promoter or 1g of oregano oil/kg have a predominance of gram-positive bacteria in the ileum. Fisher´s exact test for occurrence frequency from gram-positive bacteria showed significantly lower frequency in the ileum of negative control group compared to chickens treated with growth promoter antibiotic or supplemented with oregano oil ($p \leq 0.01$) displayed in Figure 1 and Table 2.

Table 2. Comparison between the frequencies of gram-positive bacteria, *Lactobacillus* spp. and *Enterococcus* by Fischer exact test for groups of two treatments, for a total of fourth treatments

Treatments	Gram-positive bacteria			
	Negative control	Only antibiotic	0.5g of oregano oil/kg	1.0g of oregano oil/kg
Positive control	**	n.s.	n.s.	n.s.
Negative control		**	**	**
Only antibiotic			n.s.	n.s.
0.5g of oregano oil/kg				n.s.
	Lactobacillus spp			
Positive control	**	n.s.	n.s.	n.s.
Negative control		**	n.s	**
Only antibiotic			**	n.s.
0.5g of oregano oil/kg				**
	Enterococcus spp			
Positive control	**	n.s.	n.s.	n.s.
Negative control		**	n.s	n.s
Only antibiotic			n.s.	n.s.
0.5g of oregano oil/kg				n.s.

n.s. = the frequency of treatments did not differ statistically at 5% level of probability by the test of Fischer
**= the frequency of treatments differed statistically at the level of 1% probability of the test Fischer

Besides, the negative control group had also lower growth for *Lactobacillus* spp. and higher for *Enterococcus* spp. compared with positive control group (Table 2). *Lactobacillus* spp. had higher growth in the positive control group or supplemented with 1g of oregano oil/kg diet ($p \leq 0.05$, Table 2). However, the frequency of *Lactobacillus* spp in chickens supplemented with 0.5g of oregano oil/kg diet was lower than of those supplemented with 1g of oregano oil/kg diet as well as did not differ from negative control group ($p \leq 0.05$, Table 2).

Our results corroborate Collier *et al.* (2003) that using tylosin, active against gram positives as well avylamicin, observed increase in lactobacilli. Regarding the action of plant extracts on the microbial growth, Friedman, Henika and Mandrell (2002) verified that the oregano essential oil was active against gram-negative bacteria. This effect was also observed in our results shown in Figure 1.

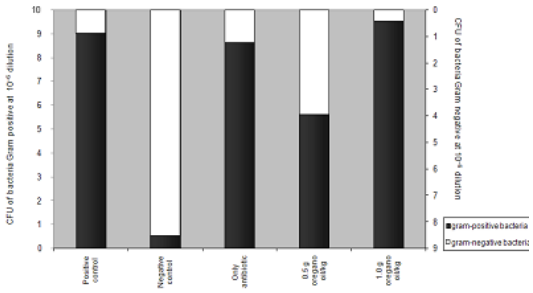

Figure1. - Colony-forming unit (CFU) of bacteria Gram positive and Gram negative Ileum of broilers at 42 days old - dilutions 10^{-6} media agar blood sodium azide / MacConkey agar.

Bona *et al.* (2012) evaluated the efficiency of a product in broiler feed containing essential oil of oregano, rosemary, cinnamon and extract of red pepper (plant compost) in the control of *Salmonella*, *Eimeria* and *Clostridium.* These authors observed that the use of the plant compost in broiler diets reduced specific lesions of *E. maxima* and *E. tenella* at 14 days after inoculation and reduced the count of colony forming units of *Clostridium perfringens* in the ceca comparing to the control group. Also, it was verified that the use of the plant compost and avilamycin decreased the excretion of *Salmonella* in poultry 72 hours after the inoculation. Fukayama *et al.* (2005) evaluated the the efficacy of oregan extract levels (0.025%, 0.050%, 0.075%, 0.100%) in the broilers diets and it was observed a decrease on the number of bacteriums in caecum as the dietary level of oregan extract increased showed the antimicrobian effect of oregan extract.

Therefore in our research it was verified that the use of oregano oil at 1g/kg diet promoted an inhibited the growth of gram negative bacteria that probably contributed to an enhancing in frequency of *Lactobacillus*. There are no studies on the action of oil of oregano on the frequency of lactobacillus in the ileum of broiler chickens.

In the present investigation, it was observed only in ileum from negative control group at 10^{-6}dilution, the presence of endospore-forming gram-positive rods hemolitic which suggested *Clostridium* spp. Therefore the diets with growth promoter antibiotic or supplemented with oregano oil, in both concentrations, had inhibitory effect on the growth of anaerobe endospore-forming gram-positive rods do tipo *Clostridium* spp.

There was not significant changes in the other population of bacteria ($p > 0.05$). Avilamycin is an antibiotic which acts mainly on *Clostridium* spp., *Staphylococcus aureus*, *Streptococcus* spp. which therefore confirm our results.The mean values of intestine relative weight are presented in Table 3.

Table 3. Relative intestine weight of broilers fed with growth promoter antibiotic or oregano oil

Diet	Intestine (g/100g of live weight)	
	19 days of age	42 days of age
Positive control	12.11 AB	5.18
Negative control	13.16 A	6.52
Only antibiotic	13.32 A	5.63
0.5 g of oregano oil/kg	12.74 A	6.53
1.0 g of oregano oil/kg	10.34 B	6.48

[A, B] Averages values within the same column with no common superscript differ significantly by the SNK test ($p < 0.05$).

At 19 days of age, it was observed that the use of 1.0 g of oregano oil/kg diet had the lowest relative intestine weight which did not differ significantly from positive control group ($p \leq 0.05$). However, there was no effect of treatments on intestine relative weight at 42 days of age ($p > 0.05$). The decrease in intestinal weight in broiler chicks denotes a lower thickness that resulted in a better feed conversion rate during 1-19 days of age (Table 2).

Thus, it has shown some effects from oregano oil on gut microbiota populations. It has been hypothesized that gut microflora decrease nutrient absorption by increasing gastrointestinal tract thickness by Apajalahti *et al.* (2004). This effect was observed in our results in animals with 19 days of age. In the studies conducted by Silva *et al.* (2009) it was verified that he broilers fed with positive control (antibiotic and anticoccidial) had the highest villous:

crypt ratio compared with the negative control that had the lowest villous:crypt ratio and the highest oocyst excretion in litter ($p < 0.05$). Also, it was observed that broilers fed with non anticoccidial agent had the highest cecal lamina propria thickeness which differ from chickens fed with anticoccidial agent in diet or supplemented with 1.0 of oregano oil kg diet-1 ($p < 0.05$).

CONCLUSION

The results showed that the dietary oregano oil exerted growth-promoting effect on broilers at 1.0g of oregano oil/kg diet by an inhibited the growth of gram negative bacteria that probably contributed to an enhancing in frequency of *Lactobacillus* spp. and an decrease in the intestinal weight in broiler chicks at 19 days of age.

Therefore, the oregano essential oil can be used as an alternative to growth promoters in animal diet.

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INFORMATIVE NOTES

The protocol of animal experimentation is in accordance to CONCEA and was approved by the Ethics Committee in Use of Animals of Espirito Santo Federal Univesity from Protocol No. 01/08.

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