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Determination of the Effects of *Z. officinale* and Propolis Extracts on Intestinal Microbiology and Histological Characteristics in Broilers

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Abstract: The purpose of this study is to explore the effects of *Z.officinale* and propolis extracts on intestine microbiology and histology in broilers as alternative growth promoters to antibiotics. One-day-old, Ross 308 male broiler chicks were used in the experiment. At the beginning of the experiment, seven treatment groups [negative control, positive control, *Z. officinale*, propolis and three different combinations of *Z. officinale* + propolis (0.5 + 0.5, 1 + 1 and 1.5 + 1.5)] were formed with similar mean weight, comprising 16 birds each. Each group was fed *ad libitum* for 42 days. Supplementation of *Z. officinale* and propolis extracts alone or in combination reduce Coliform bacteria ($p<0.05$) compared to negative control group. The highest numbers of total mesophilic aerobic bacteria was identified in the group with (1:1) combination of *Z. officinale* + Propolis ($p<0.05$). The high amount of such bacteria in this group can be attributed to the lack of Coliform bacteria and decreasing tendency in *E. coli* content. The desired stimulating effect on lactic acid bacteria was observed in all treatment groups compared to negative control group. *Z. officinale* and Propolis extracts and their combinations significantly improved intestinal villi length ($p<0.05$) compared to negative control group. These affirmative findings indicate likely improvement in performance parameters of animals in treatment groups. As a result; *Z. officinale* and Propolis extracts could have a great potential to promote broiler growth.

Key words: Broiler, *Z. officinale*, propolis, intestinal microbiology, intestinal histolohgy

INTRODUCTION

Aromatic plants have traditionally been used as medicine for treatment of diseases. The utilization of aromatic plants as antimicrobial growth promoters has not been much taken into consideration in modern animal feeding practices. There are ongoing studies for identification of existing benefits of plant extracts and their future utilization as valid alternatives (Kamel, 2000). The primary goal of plant extraction is to purify the plant from unnecessary substances and to obtain the main active substances in pure state (Wheeler, 1993). One of the well-known main specific effects of plant extracts is their antimicrobial activities. Indeed, there is plenty of scientific literature with laboratory research that have proved the antibacterial, antifungal and antiviral effects of many extracts against poultry and/or food-borne pathogens (Kamel, 2000). The antimicrobial activities of plant extracts are explained by some mechanisms. For instance, the antimicrobial activity of isothiocynates, which is derived from onion (*Allium cepa*) and garlic (*Allium sativum*), is explained by its ability of inactivation of extracellular enzymes (Brul and Coote, 1999). However, the antibacterial effect of many essential oils is associated with their cell-wall damaging activity. This activity affects electron exchange, ion density, protein synthesis, phosphorylation steps and enyzme-based direct reactions. All these lead to loss of chemical osmotic balance in bacteria (Ultee *et al*., 1999; Cox *et al*., 2000; Dorman and Deans, 2000). For instance, Helander *et al*. (1998) revealed that both carvacrol and thymol inhibited the growth of *E. coli* and carvacrol increased cell wall permeability for H^+ and K^+ ions.

Plants and plant extracts are effective mainly on the digestive system of animals. They function either by wiping out the pathogenic microflora in the digestive system or increasing the concentration of microbial population in the digestive system that contributes to improved digestion and absorption of nutrients (Wenk, 2000). Animals, poultry in particular, are very sensitive to pathogenic bacteria such as *Escherichia coli, Salmonella ssp, Clostridium perfringens* and *Campylobacter sputorum*. Anbiotics which for long time have been used as growth promoters in poultry rations stabilize the intestinal microbial flora and improve growth performance by inhibiting some specific intestinal pathogens (Gunal *et al*., 2006). Some bioactive derived from fungus and higher plants have been reported to kill pathogenic bacteria species in chickens while increasing beneficial bacterium species such as lactic acid and bifidobacteria. The stimulation of such beneficial bacteria generates an effective protection against pathogenic microorganisms and a balanced

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intestinal microflora (Vidanarachchi *et al*., 2006). The significant decrease in the numbers of pathogenic microorganisms is accompanied by the increase in the amount of mucous IgA protecting the intestinal mucosa against pathogenic microorganisms. Furthermore, while the villi length in small intestine increases, crypt cell density has been reported to decrease. Shorter villis and more dense crypts have been associated with the existence of toxins in intestinal flora. Pathogens also lead to morphological changes in intestines as well as physiological changes. They interfere with the absorption of nutrients and increase secretion. As a result, they lead to diarrhoea, decrease in resistance to diseases and ultimately, decrease in efficiency (Nabuurs *et al*.,1993).

Another increasingly important recent natural product is propolis. Propolis is a glue-like substance that honey bees collect from plant seedlings and buds. It is obtained as a result of the biochemical alteration of the resinous materials and plant secretions by the enyzmes secreted from the glands of the bees. It has a colour ranging from dirty yellow to dark brown, a strong and nice odor, is water-insoluble and semi-solid in room temperature (Hepsen *et al*., 1996; Sahinler, 2000). Since the chemical composition of propolis is highly complex and its composition varies according to the plant, region, season and colony; its colour, odor and medical characteristics vary accordingly (Kutluca, 2003). The composition of pure propolis is generally composed of 50% resin-vegetal balsam, 30% beeswax, 10% essential-aromatic oils, 5% pollen and 5% other organic substances (Kumova *et al*., 2002; Dodologlu *et al*., 2003; Silici, 2003).

In addition to its content of chemical compounds, propolis is highly nutritious due to its content of vitamins B_1, B_2, B_6, C and E as well as minerals such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe. Propolis also contains some enzymes like dehydrogenase, glucose-6-phosphatase, Adenosine Triphosphate (ATP) and acid phosphatase (Tikhonov and Mamontova, 1987; Kumova *et al*., 2002; Yilmaz *et al*., 2003). Propolis collected from hive is raw and it has to be subjected to purification prior to utilization. It has to be solved in ethanol. 70% ethanol solution is used for medical utilization of propolis (Gencay and Sorkun, 2002).

The present literature showed that thers is no information about growth promoting and antimikrobioklogical and histological effects of *Zingiber officinale* and animal-borne natural substance as Propolis in broilers. However, the extracts obtained from the aromatic plant *Zingiber officinale* and animal-borne natural substance as Propolis have particular importance due to their content of effective substances. The purpose of this study is therefore was undertaken to determine the effects of *Z. officinale* and propolis extracts on intestinal microbiology and histological characteristics in broilers due to the antibacterial properties of these extracts.

MATERIALS AND METHODS

105, one-day-old male broiler chicks (Ross 308) were divided into seven treatment groups of 15 birds each. The groups were as follows: 1. Negative control (not including antibiotic), 2. Positive control (including antibiotic), 3. *Zingiber officinale* (240 ppm), 4. Propolis 1000 ppm, 5. *Zingiber officinale* 120 ppm + Propolis 500 ppm combination, 6. *Zingiber officinale* 240 ppm + Propolis 1000 ppm combination, 7. *Zingiber officinale* 360 ppm + Propolis 1500 ppm combination. The composition of the basal diet is presented in Table 1. Birds were given a starter diet to 10 d, a grower diet from 11 to 21 d, thereafter a finisher diet to 42 d. Each group was fed *ad libitum* its own diet for a period of 42 days. 24 h light was provided per day. *Zingiber officinale* essential oils were purchased from Ege Lokman San. Tic. Company, Manisa, Turkey. Propolis was taken from Erciyes University, S. Cikrikcioglu Junior Technical College-KAYSERÝ. Major components of *Z. officinale* essential oil and propolis were analysed using MSGC, the composition is given in Table 2.

At the end of the experimental period five birds of similar body weight from each treatment group were slaughtered to determine intestinal villi length. Intestinal samples for villus were taken from the point connect with jejunum of the Merckel's diverticulum and placed into bottles with 10% formalin solution for morphological analysis. From each intestine sample, one portion was cut perpendicular to longitudinal axis of the intestine and embedded in paraffin wax. Transverse sections were cut (3 μm) and stained with hematoxylineosin method to determine villus height by light microscopy observation at 4x. By the use of BAB Bs200Doc image processing and analyzer system installed in light microscope, overall section photos were obtained in 4x objective and villi photos (Fig. 1) were obtained in 10x objective. Five villi were measured per sample using BS200doc analysis software. The feaces from three birds of similar body weight were collected to determine intestinal microflora contents. After slaughter, feaces samples from jejunum part was taken immediately and diluted with 1:10 deionize water followed by culture analyses. During counting, three agars were used, Plate Count Agar for total mesophilic aerobic bacteria, VRB Agar for Coliform group and MRS Agar for Lactic acid. Additionally, Fluorocult Lauryl Sulfate Broth (Merck) Agar and Most Probable Number (MPN) method was used for coliform and *E. coli* counts (Halkman *et al*.,1994; Merck, 1998).

Table 1: The ingredient and chemical composition (g/kg) of starter, grower and finisher diets

Ingredients	Starter diet (1-10 d)	Grower diet (11-21 d)	Finisher diet (22-42 d)
Maize	471.09	493.19	553.13
Full-fat soyabean	180.00	170.00	190.00
Soya bean meal (%46 CP)	156.78	130.14	89.77
M.Gluten meal (%55 CP)	70.88	48.73	13.15
Polutry offal meal (%52 CP)	40.00	45.00	40.00
Meat-bone meal (%32 CP)	28.98	33.23	26.86
Fish meal (%70 CP)	25.00	39.10	42.72
Crude cotton oil	12.05	29.69	34.50
Dicalcium phosphate (%18 P)	3.00	-	0.49
Salt	1.00	-	-
Soda	1.58	2.55	2.56
Lysine	2.53	1.53	1.00
Methionine (Alimet)	2.11	1.84	2.32
Vitamin premix[1]	3.00	3.00	2.00
Mineral premix[2]	2.00	2.00	1.50
Total	1000.00	1000.00	1000.00
Analyses			
ME (kcal/kg)	3028.52	3175.00	3260.00
Dry matter	88.56	88.69	88.54
Crude protein	25.77	24.66	21.80
Ether extract	8.17	10.00	10.70
Crude cellulose	3.49	3.35	3.38
Crude ash	6.35	6.17	5.72
Lysine (%)	1.52	1.43	1.29
Methionine + Cystine	1.06	1.00	0.94
Calcium	1.01	1.04	0.91
Available phosphorus	0.48	0.47	0.45

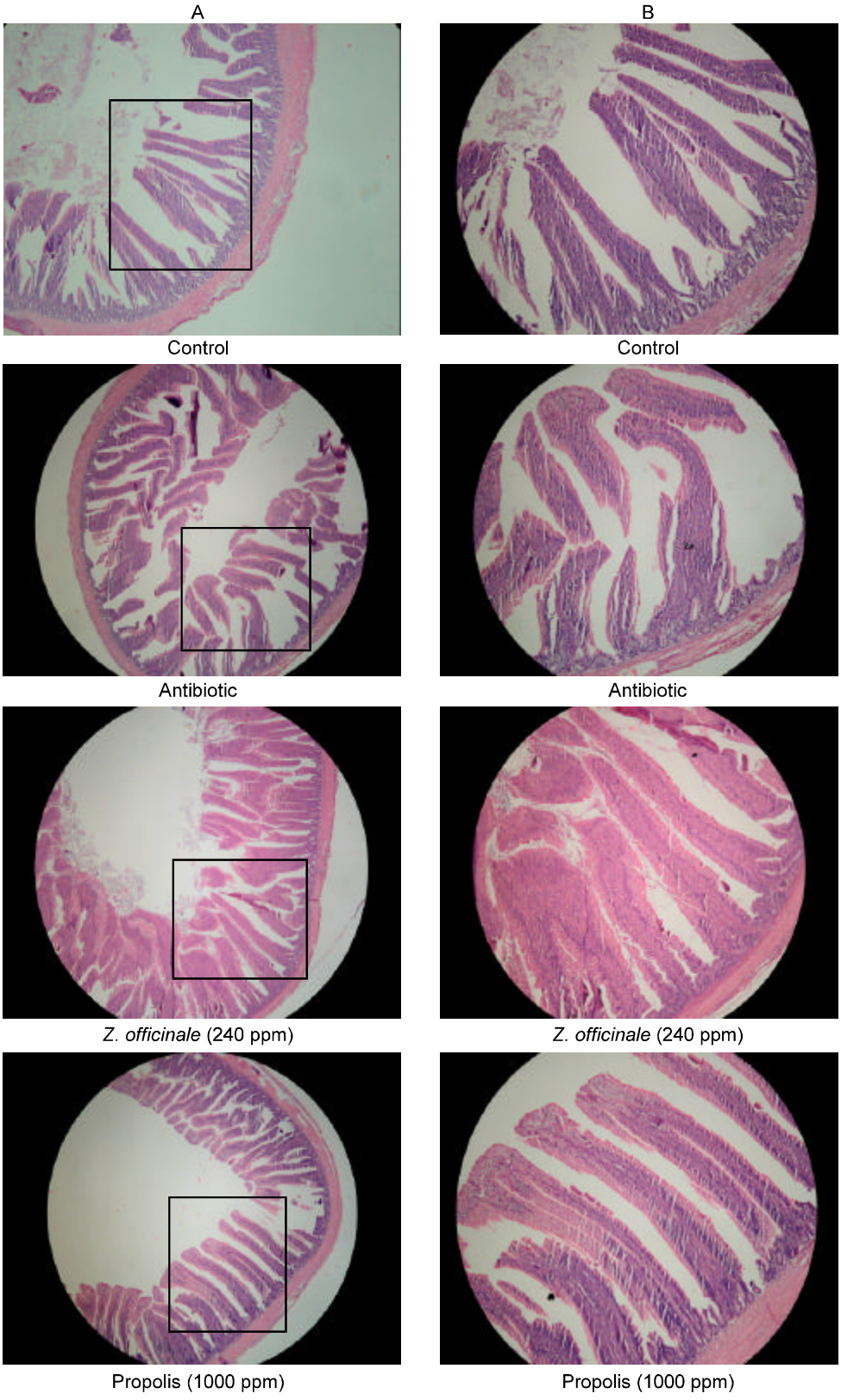
[1]Vitamin premix per 2.5 kg of premix: 12 000 000 IU Vitamin A. 3 500 000 IU Vitamin D3. 100 g Vitamin E. 3 g Vitamin K3. 2.5 g Vitamin B1. 6 g Vitamin B2. 25 g Niacin. 12 g Ca-D-Pantotenat. 4 g Vitamin B6. 15 mg Vitamin B12. 1.5 g Folic Acid. 150 mg D-Biotin. 100 g Vitamin C. 450 g Choline Chloride.

[2]Mineral premix per kg: 100 mg Mn; 25 g Fe; 65 g, Zn; 15 g, Cu; 0.25 g, Co; 1 g, I; 0.2 g Selenyum

Table 2: *Zingiber officinale* and propolis essential oil and major components (%)

Zingiber officinale	%	Propolis	%
Cis 2-Nonenal	1.75	**Bilesikler components**	
(E,E) 2,4-Decadienal	13.79	**Flavonoids**	
Ar-Curcumene	8.93	Chrysin	5.33
Zingiberene	15.77	Naringenin	2.67
α-Farnsene	3.27	2-methoxy-4-vinylphenol	0.47
Valancene	1.29	4-vinylphenol	0.44
β-Bisavolene	7.68	Hexanoic acid	0.64
β-Sesquiphellandrene	11.97	4-pentenoic acid	0.25
1,3,5-Cyclooctatriene	0.70	2-Propenoic acid	0.38
Zingerone	4.63	3-hydroxy-4-methoxy cinnamic acid	0.56
Viridiflorol	0.72	Hexadecanoic acid	1.21
β-Copanen-4,α ol	10.98	9-Octadecanoic acid	0.55
Linoleic Asit	0.50	**Alifatic, aromatic and fatty acids**	
Oleic Asit	0.62	Ferulic acid	2.26
n-Hekza Dekonoik Asid	1.04	**Esters**	
Retinol	0.54	Benzyl cinnamate	1.35
Monopalmitin	3.19	**Terpens**	
Retinol Acetate	0.22	d-Limonene	0.28
Stearoik Asit	4.06	β-eudesmol	1.00
Linoleyl Choloride	4.19	α-eudesmol	0.89
Squalene	0.38	**Aldehids, ketons and others**	
3-(6-Hydroksi, 3,7 Dimethy-octa 2,7, dieniyl)-4-Methozy fenol	1.73	Crysophanol	22.07
Octadecane, 3-ethy-5-(2-ethylbutryl)	0.71	4-H-1-benzopyran-4-one	13.51
Lucerin 2	0.42		
n-Heptacosane	0.91		

The data obtained in the experiment were analysed using the GLM procedure of SAS (1987) and treatment means were separated using Duncan's New Multiple Range Test


A
B
Control
Control
Antibiotic
Antibiotic
Z. officinale (240 ppm)
Z. officinale (240 ppm)
Propolis (1000 ppm)
Propolis (1000 ppm)

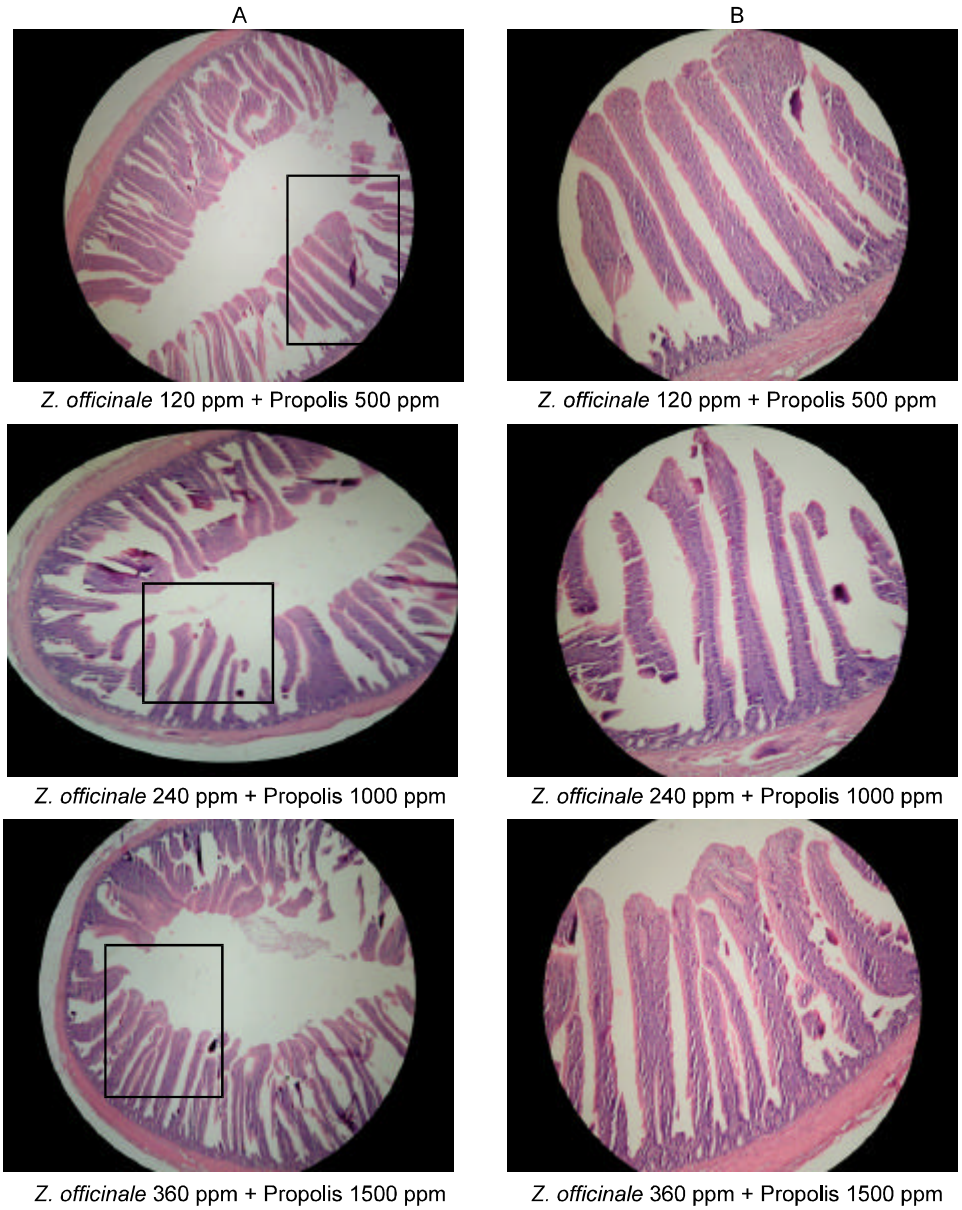


Fig. 1: Deneme 2 muamele gruplarina ait jejunum villi görüntüleri (A sutunu 4x, B sutunu 10x)

RESULTS AND DISCUSSION

In accordance with the findings of the study, the populations of total mesophilic aerobic, coliform and lactic acid bacteria in small intestine (jejunum) are given in Table 3 and *E. coli* content is presented in Table 4. The measurements on jejunum content of the small intestine have revealed that while the type of treatment did not have a significant effect ($p>0.05$) on the existence of host lactic acid bacteria, it significantly affected ($p<0.05$) the existence of total mesophilic aerobic and coliform bacteria. Coliform group bacteria in all treatment groups have significantly decreased with respect to control group. The highest total mesophilic aerobic bacteria population was identified in the group with (1:1) combination of *Z. officinale* and propolis. The high amount of such bacteria in this group can be attributed to the non-existence of coliform group bacteria. Additionally, *E. coli* content was identified to have a decreasing tendency in the groups with *Z. officinale* and propolis combinations.

Z. officinale and propolis extracts supplemented in the ration both separately and in combination proved to stimulate lactic acid bacteria and significantly decrease pathogenic bacteria such as total mesophilic aerobic, coliform and *E. coli*. This effect is due to the ability of essential oils in terms of inactivation of extracellular

Table 3: Effect of *Z. officinale* essential oil and propolis supplemental on bacterial counts in jejunum digesta of broiler chicks at 42 days old

	Bacteria types		
Treatment groups	Total mesophile aerob	Coliform group bacterias	Laktic acid
	(cob/g)		
Negative control (No Additive)	$45x10^4$b	$1500x10^2$a	$1.2x10^6$
Pozitive control (Antibiotic)	$24x10^4$b	$100x10^2$b	$4x10^6$
Z. officinale 240 ppm	$6.8x10^4$b	$160x10^2$b	$4x10^6$
Propolis 1000 ppm	$1.9x10^4$b	$52x10^2$b	$3.6x10^6$
Z. officinale 120 ppm + Propolis 500 ppm	$42x10^4$b	$3x10^2$b	$3.7x10^6$
Z. officinale 240 ppm + Propolis 1000 ppm	$680x10^4$a	$1x10^0$b	$3.5x10^6$
Z. officinale 360 ppm + Propolis 1500 ppm	$60x10^4$b	$210x10^2$b	$4.1x10^6$
SED	31410.07	8573.74	23805.73
Significance (P=)	0.0001	0.0001	0.9217

*Means within same column having different letters are significantly diferent ($p<0.05$). SED: Standard error of diference between means

Table 4: *E. coli* Analysis reults in jejunum (EMS/ml)

Treatment groups	Sample No.	*E. coli* (EMS/ml)
Negative control (No Additive)	1	>1100
	2	>1100
	3	>1100
Positive control (Antibiotic)	1	>1100
	2	>1100
	3	>1100
Z. officinale (240 ppm)	1	>1100
	2	>1100
	3	>1100
Propolis (1000 ppm)	1	>1100
	2	1100
	3	1100
Z. officinale 120 ppm	1	240
+ Propolis 500 ppm	2	1100
	3	240
Z. officinale 240 ppm	1	>1100
+ Propolis 1000 ppm	2	>1100
	3	1100
Z. officinale 360 ppm	1	>1100
+ Propolis 1500 ppm	2	460
	3	>1100

enzymes and their antibacterial properties which lead to death of bacteria by decreased pH of the medium and damage to cell wall structure. As known, phenolic structures denature the proteins in the cell wall of the bacteria and increase cell wall permeability. The corrupted permeability of the cell wall induces the release of the intracellular fluid, which, consequently, kills the bacteria (Kutlu, 1999). Similarly, other researchers also stated that the antibacterial effect of many essential oils is attributed to their cell-wall damaging activity. This activity affects electron exchange, ion density, protein synthesis, phosphorylation steps and enyzme-based direct reactions. All these lead to disturbance of osmotic balance in bacteria (Ultee *et al*., 1999; Cox *et al*., 2000; Dorman and Deans, 2000).

Bakaowski (2001) stated that the increase in live body weight induced by plant extracts is associated with the protective effect of phytogenic feed additives on intestinal flora against invading micro-organisms. Bruggeman *et al*. (2002) revealed that plant extracts performed well against *E. coli* in the *in vitro* and *in vivo* studies on poultry and pigs. The study of Avci (2004) showed that in broilers, plant extracts had an effect on ileum *enterobactericeae* population and the lowest numbers of enterobacteria was identified in fennel group. Guo *et al*. (2004) reported that plant extracts led to a decrease in the numbers of harmful bacteria (*Bacteroides* sp. ve *E. coli*) in the caecum while at the same time increasing the numbers of beneficial bacteria (*bifidobacteria ve lactobacilli*). In the study of Dalkilic *et al*. (2005), the highest numbers of coliform bacteria were identified in the control group compared to the plant extract groups; the extent of antimicrobial effect increased with the increase in the amount of essential oils and Thyme + Anise 400 group had a superior antibacterial effect on cecal coliform group bacteria compared to that of antibiotics. Sarica *et al*. (2005) reported that thymol and garlic significantly reduced the numbers of *E. coli* in small intestine. Lotfy (2006) stated that the bacteria types that are resistant to antibiotics were sensitive to propolis and propolis was effective against *S. aureus* ve *S. epidermis* bacteria in chickens under *in vitro* conditions. Similarly, Vidanarachchi *et al*. (2006) revealed that plant extract additives increased the numbers of lactic acid bacteria in the ileum and cecum of broilers while significantly reducing the numbers of total anaerobic, coliform and *C. perfringes* bacteria. All these findings are in agreement with our results. On the other hand, Demir *et al*. (2003), reported that plant extract groups including oregano, du-sacch, quiponin, garlic and thymol had no effect on cecum *E. coli* content. The findings of these researchers are not in agreement with the findings of our study. The inconsistency between the findings of two studies are likely to stem from the different extracts used in each study as alternative to antibiotics as well as the different sections employed for bacterium count.

The data related to intestinal (jejunum) villi lengths and pH values are given in Table 5. *Z. officinale*, propolis and the combination of the two extracts are determined to

Table 5: Effect of *Z. officinale* essential oil and propolis supplemental on villus height and pH in jejunum of broiler chicks at 42 days old

Treatment groups	Villi height (μm)	pH
Negative control (No Additive)	577.48c	6.27
Positive control (Antibiotic)	765.20a	6.12
Z. officinale 240 ppm	636.12bc	6.50
Propolis 1000 ppm	706.00ab	6.06
Z. officinale 120 ppm + Propolis 500 ppm	701.88ab	6.11
Z. officinale 240 ppm + Propolis 1000 ppm	746.08a	6.24
Z. officinale 360 ppm + Propolis 1500 ppm	738.76a	6.01
SED	9.91	0.06
Significance (P=)	0.0001	0.3279

*Means within same column having different letters are significantly diferent ($p<0.05$). SED: Standard error of diference between means

have a significant affect on intestinal villi lengths ($p<0.05$). While the differences among intestinal jejunum pH values were insignificant ($p>0.05$), in the groups supplemented with propolis and propolis + *Z. officinale* combination, jejunum pH value was recorded to have a numerically decreasing tendency.

The findings of our study suggest that intestinal villi length significantly improved in the groups supplemented with antibiotic, *Z. officinale*, propolis and *Z. officinale* + propolis combination with respect to the control group. Our results support the findings of Bruggeman *et al.* (2002) about the improving effects of plant extracts on digestive system villi morphology in poultry and pigs; the findings of Catala *et al.* (2004) about the significantly improving effects of plant extracts on intestinal villi lengths and villi surface areas; the findings of Vidanarachchi *et al.* (2006) about the decreasing effects of plant extracts on ileum pH values; the findings of Tekeli *et al.* (2006) obtained with 240 ppm *Z. officinale* and Tekeli *et al.* (2008) obtained with 1000 ppm propolis supplements and the findings of Gunal *et al.* (2006) about the significantly improving effects of probiotic supplements on ileum and jejunum villi length. The decrease in jejunum pH content, the suppression of pathogenic bacteria species and the stimulation of beneficial bacteria like lactic acid are thought to contribute in the formation of a balanced intestinal microflora. The jejunum villi of test groups are displayed in Fig. 1 obtained in 4x and 10x objectives. These natural supplements considered as alternative growth promoters to antibiotics showed a positive effect on intestinal histological and physiological characteristics. This improving effect is attributed to the stimulatory and promotive effects of the effective ingredients in the plant extracts on the juices and enzymes in the digestive system. This improves the morphology of the digestive system. The short-chain fatty acids in the extracts lead to suppression of pathogenic bacteria in the digestive system by stimulating intestinal epithelial cells since shorter villis and more dense crypts have been associated with the existence of toxins in intestinal flora (Nabuurs *et al.*, 1993). On the other hand, Demir *et al.* (2003) reported that the crypt depth in ileum significantly decreased in the groups supplemented with garlic and thymol compared to those groups supplemented with antibiotic, oregano and du-sacch ($p<0.05$). In the study of Gunal *et al.* (2006), ileum and jejenum villi height have significantly been improved by propolis supplementation with respect to the group supplemented with antibiotic and that supplemented with the product "Genex" (a mixture of plant extract + organic acid). However, the villi lengths achieved by *Z. officinale*, propolis and *Z. officinale* + propolis supplementations in our study were found to be higher than the villi length obtained by Genex. This difference is possibly due to the organic acid of Genex in addition to plant extracts.

The results obtained in the experiment revealed that:

1 In the groups supplemented separately with 240 ppm *Z. Officinale*, with 1000 ppm propolis extract and the combination of these two extracts, the numbers of beneficial bacteria (lactic acid) in the intestine (jejenum) of broilers increased accompanied with decreased numbers of harmful bacteria (coliform and *E. coli*) and increased villi length.
2 *Z. officinale* and propolis extracts had a stimulatory effect on lactic acid bacteria and decreasing effect on harmful bacteria such as coliform and *E. coli*. These effects are explained by the ability inherent in essential oils to inactivate extracellular enzymes and by their antibacterial effects which lead to damage in the cell wall structure and death of harmful bacteria (Brul and Coote, 1999). Shorter villi have been associated with the presence of toxins in intestinal flora.

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