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Effect of dietary supplementation with extracts of rosemary, olive leaves, pine bark and quercetin on selected performance indices of broiler chickens and microbiological status of their ileum[1)]

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Summary

A total of 180 1-day-old male Hubbard Flex broiler chickens were used in a 32-day model experiment to determine the effects of dietary supplementation with quercetin (Q) and with polyphenolic extracts of rosemary (RO), olive leaves (OL) and pine bark (PB) on the performance of the birds and the microbiological status of their ileum. The chickens were randomly allocated into 9 groups: the control group (with 6 replicates, 6 birds per cage) and 8 treatment groups (with 3 replicates in each, 6 birds per cage), and fed *ad libitum* throughout the experimental period with a basal isoenergetic and isoprotein control diet or with the same basal diet containing two concentrations of RO, OL and PB extracts (2.50 and 5.00 g/kg), and Q (0.25 and 0.50 g/kg). The body weight gain (BWG) and the feed conversion ratio (FCR) were determined during the experiment. At day 32, two randomly selected birds from each cage were slaughtered, and 5-centimetre-long pieces of the ileum beginning from the Meckel's diverticulum were collected to analyze the number of microorganisms in the intestinal content. Chickens' weight gain and FCR were not affected by the OL-, PB- and Q-enriched diets, but supplementation with RO significantly ($P < 0.05$) impaired FCR. BWG was significantly ($P < 0.05$) reduced when chickens were fed with mixtures containing 2.50 and 0.25 g/kg of the polyphenolic additives. The number of CFUs of intestinal microorganisms was not significantly affected ($P > 0.05$) by the diet modification. However, a large decrease ($P > 0.05$) was observed in the CFUs of coliform bacteria (up to 96%), *E. coli* (up to 93%), *Lactobacillus* spp. (up to 89%), molds and yeasts (up to 95%) and anaerobic *Clostridium* spp. (up to 52%) in the ileum content of chickens supplemented with the additives containing polyphenols.

Keywords: polyphenolic extracts, quercetin, DPPH, broiler chickens, performance, microbiological status of ileum

Antibiotic growth promoters (AGPs) have long been used in animal nutrition to improve growth performance and feed efficiency. However, since January 1, 2006, the use of AGPs has been banned by the European Commission because of the potential increase in the antibiotic resistance of animal and human pathogens (28). As a result of this regulation, reduced animal growth performance and higher feed consumption have been observed (12). To improve these two economically important indices, as well as animal health, numerous studies have been carried out to find alternative phytogenic substances, such as herbs, essential oils and plant extracts, especially those rich in polyphenols.

Polyphenols belong to a large group of secondary plant metabolites and are known as strong antioxidants. They have antimicrobial properties and play an important role as anti-inflammatory (9) and antimutagenic

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(14) agents. Recently, numerous studies involving extracts of rosemary, olive leaves and pine bark have been carried out *in vitro* in order to determine their antimicrobial potential. Travassoli and Djomeh (34), and Abramovič et al. (2) showed, that rosemary extracts show strong antimicrobial activity against *Escherichia coli*, *Salmonella infantis*, *Leuconostoc mesenteroides*, *Lactobacillus delbruekii*, *Streptococcus aureus*, *Campylobacter jejuni*, and yeasts. Extract from olive tree leaves has also been shown to inhibit the growth of *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Cryptococcus neoformans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* (22). A wide range of antimicrobial activity of pine bark extract against *Escherichia coli*, *Clostridium perfringens*, *Salmonella* spp., *Candida albicans*, *Aspergillus oryzae* and *Penicillium funicusolum* was demonstrated by Ahna et al. (4) and Torras et al. (33).

Although complex mixtures of polyphenols generally exhibit higher antimicrobial activity than their individual components (20), highly purified plant extract constituents can also potentially be used as effective growth promoters. The best example is quercetin (3,5,7,3',4'-pentahydroxyflavone), present in onion and apple skin. A strong antimicrobial activity of quercetin was observed especially against *Sarcina maxima*, *Micrococcus kristinae*, *Klebsiella pneumoniae* and *Aspergillus flavus* (1). This flavonol also shows bacteriostatic properties against *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Escherichia coli* (11, 18).

Therefore, the objective of this model study was to determine the effects of dietary supplementation with three commercial plant extracts containing polyphenols, namely, extracts from rosemary (RO), olive leaves (OL) and pine bark (PB), and with a pure polifenolic (flavonol) compound, quercetin (Q), on the microbiological status of the broiler chicken's ileum content and on selected performance indices of broiler chickens.

Tab. 1. Composition of experimental diets fed to broilers

Ingredients (g/kg)	Control diet (C)	2.50 g/kg RO, OL, PB	5.00 g/kg RO, OL, PB	0.25 g/kg Q	0.50 g/kg Q
Maize	250.0	247.5	245.0	249.75	249.5
Wheat	312.9	312.9	312.9	312.9	312.9
Soy bean meal	346.7	346.7	346.7	346.7	346.7
Canola oil	49.1	49.1	49.1	49.1	49.1
Chalk	19.9	19.9	19.9	19.9	19.9
Dicalcium phosphate	8.6	8.6	8.6	8.6	8.6
Sodium chloride	2.8	2.8	2.8	2.8	2.8
Premix DKA s/g*	10.0	10.0	10.0	10.0	10.0
Extract**	0.00	2.50	5.00	0.00	0.00
Quercetin***	0.00	0.00	0.00	0.25	0.50
Energy value (MJ) and essential nutrients (g/kg) of experimental diets					
ME, MJ/kg	12.50	12.47	12.43	12.50	12.49
Dry matter g/kg	894.24	894.42	894.59	894.26	893.80
Crude protein g/kg	220.00	219.77	219.53	219.98	219.95
Crude fiber g/kg	29.91	29.84	29.77	29.90	29.90
Crude fat g/kg	72.49	72.30	72.03	72.48	72.47
Crude ash g/kg	30.90	30.86	30.82	30.90	30.89
Nitrogen free extract g/kg	540.94	541.56	542.19	541.00	539.66
Ca g/kg	9.40	9.39	9.38	9.40	9.40
P available g/kg	4.30	4.27	4.25	4.29	4.29
Na g/kg	1.60	1.59	1.59	1.60	1.60
Concentration of active substances in RO, OL, PB and Q preparations added to diets (g/kg)****					
Carnosol	0.00	0.16	0.31	0.00	0.00
Oleuropein	0.00	0.50	1.00	0.00	0.00
Pycnogenol	0.00	2.49	4.98	0.00	0.00
Quercetin	0.00	0.00	0.00	0.24	0.47

Explanations: *content of additives added in the premix to 1 kg of mixture: $CaCO_3$ – 1.40 g, P – 0.75 g, S – 250 µg, Mn – 90 mg, J – 800 µg, Zn – 80 mg, Fe – 50 mg, Co – 400 µg, Se – 250 µg, retinol – 3.75 g, cholecalciferol – 75 µg, α-tocopherol – 50 mg, menadione – 3 mg, thiamin – 2 mg, riboflavin – 6 mg, pyridoxine hydrochloride – 35 mg, cyanocobalamin – 20 µg, pantothenic acid – 15 mg, biotin – 600 µg, nicotinic acid – 50 mg, folic acid – 1.5 mg, choline – 600 mg, phytase, coccidiostat – salinomycin; **concentrations of extracts of RO (rosemary), OL (olive leaves) and PB (pine bark); ***concentration of quercetin (3,5,7,3',4'-pentahydroxyflavone); ****calculated according to manufacturer's data

Material and methods

Animals and diets. A total of 180 one-day-old male Hubbard Flex broiler chickens (mean body weight: 39.9 ± 1.1 g) were randomly allocated to eight dietary treatments (each in three replicates) and to a control group (with six replicates). The chickens were reared for 32 days in battery cages with six birds per cage (a replicate). The ambient temperature during the experiment was gradually reduced from at 34°C on day 1 to 23°C on day 32, according to Hubbard Flex Broilers breeding recommendations. The lighting programme throughout the study consisted of 18 h of light and 6 h of darkness. The birds had unlimited access to feed and drinking water (nipples). The management of the birds was in compliance with regulations of the European Union and Ethical Commission (10). All procedures involving animals were approved by the Local Ethics Commission for animal experiments (approval no. 16/2010, 18.01.2010).

The broilers were fed with diets in a mashed form, based on maize, wheat and soybean meal (Tab. 1). Crude protein content in the basal mixture equaled about 220 g/kg. The energy value was then

calculated at 12.5 MJ/kg using WPSA (37). The birds in the control group were supplied with a basal feed mixture (control, C), while the experimental groups were given the basal diet supplemented with polyphenol-rich extracts of rosemary – RO (groups II and III), olive leaves – OL (groups IV and V) and pine bark – PB (groups VI and VII) and with quercetin – Q (groups VIII and IX). Two concentrations of each of the above additives were added to the diet, i.e. 2.50 and 5.00 g/kg for RO, OL and PB, and 0.25 and 0.50 g/kg for Q (Tab. 1). The lower concentrations of Q were chosen due to the fact that its scavenging activity is 2-12 times that of the other additives tested (Fig. 1). The vitamin premix added to the diets contained phytase and salinomycin as coccidiostat.

Experimental measurements. The body weight (BW) of the chickens of each replicate (pen) was controlled on days 1, 14, 27 and 32. The feed intake was recorded for days 1-14, 15-26 and 27-32 and calculated for the whole experimental period, as well. Mortality and other losses were recorded throughout the study. At the end of the experiment, two birds from each replicate were randomly selected and slaughtered (they were fed about 12 h before the slaughter). Subsequently, 5-centimetre-long pieces of the ileum beginning from the Meckel's diverticulum were collected to determine the number of selected microorganisms in the ileum content.

Radical scavenging ability. Commercial, standardized extracts of RO (6 g/kg of carnosol), OL (200 g/kg of oleuropein) and PB (997.7 g/kg of pycnogenol) were obtained from PIBS Foundation (Poznań, Poland). Quercetin (≥ 95%, HPLC) was purchased from Sigma Aldrich (Germany). The antiradical activity against 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radicals was analyzed in methanol solutions of RO, OL and PB extracts and in Q at 516 nm according to Sierżant et al. (30). The capacity for scavenging the synthetic radical was expressed as IC_{50}^{DPPH} (half maximal inhibitory concentration) and given in mg/L. Analyses were performed in five replicates.

Microbiological analyses. The analyses of ileum content collected from 32-day-old chickens were carried within two hours of slaughter. One gram of gastrointestinal content was homogenized for 60 s in a Stomacher Lab-Blender 400, followed by the preparation of 10-fold dilutions in sterile Peptone Water. The number of selected microorganisms (expressed as CFU – colony forming units) was determined according to specified standards: total number of coliforms (26), *Escherichia coli* (27), *Lactobacillus* spp. (23), yeasts and molds (25), *Clostridium perfringens* (26). The analyses were performed in triplicate.

Other analytical methods used for diets. The chemical composition of feed components and excreta was analyzed according to AOAC (5): dry matter (DM) was determined using a Zalmed SML 32/250 dryer, crude ash was determined using a muffle furnace, nitrogen was determined by the Kjeldahl method with a Kjeltec 2300 Foss Tecator (Hillerod, Denmark); crude protein (CP) was measured as 6.25 N, crude fat (CFA) was determined by ether extraction with a Buchi Extraction System B-811; crude fiber (CF) was determined using a Foss Fibertec 1020 (Hillerod, Denmark).

Statistical analyses. The data obtained were analyzed statistically by two-way ANOVA using the StatSoft Statistica® Software (31) and then by Tuckey's Multiple Comparison Test (factorial designs with a separate control group). Differences between the treatments for all parameters were tested according to the following statistical model: $y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$, where y_{ijk} – variance associated with parameter, μ – overall mean, α_i – treatment effect, β_j – effect of concentration of extract in diet, $(\alpha\beta)_{ij}$ – interaction effect, e_{ijk} – impact of specific factors. The results for the antiradical activity of the extracts and Q were analyzed by one-way ANOVA and Tuckey's Multiple Comparison Test.

Results and discussion

Antiradical activity. The antiradical activity of the plant extracts and quercetin is shown in Figure 1. Quercetin exhibited a very strong antioxidant potential (3.17 mg/L), which was about twice as high as that of the PB extract (6.26 mg/L). The capacity of the OL and RO extracts for neutralizing the synthetic radical was 8-10% that of the pure flavonoid (Q) and about 17-20% that of the PB extract ($P < 0.01$).

Performance indices of broiler chickens. The average body weight gain (BWG) and feed conversion ratio (FCR) of broilers fed diets supplemented with RO, OL, PB extracts and Q are presented in Table 2. No significant differences in mortality were observed between the treatment groups throughout the experiment (1.05% in 0.50 g/kg of Q versus 0% in other groups).

The average BWG of chickens from 1 to 32 day of age varied from 1.439 g (RO) to 1.556 g (Q), and it was not affected ($P > 0.05$) by the supplementation of polyphenolic additives. The birds receiving diets with the lower amount of plant extracts and quercetin (0.25% and 0.025%, respectively) showed significantly ($P < 0.05$) lower BWG compared to those that received the control mixture or diets supplemented with the higher concentration of phytogenic additives (Tab. 2). These results were contrary to those of Park (21), who reported that the addition of 70 mg/kg PB to the broiler diet significantly ($P < 0.05$) increased

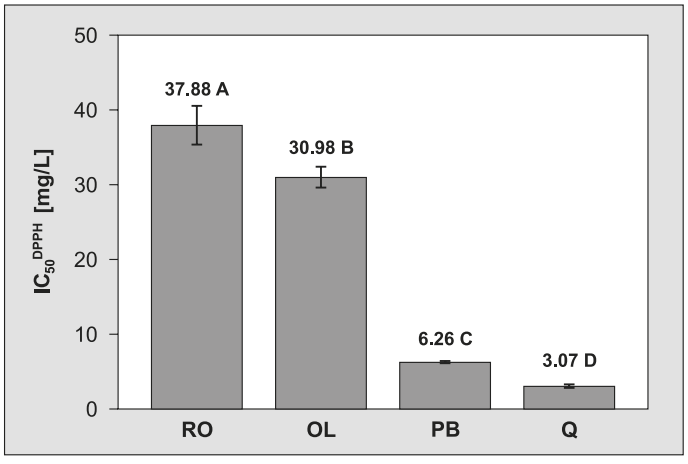

Fig. 1. Antiradical activity of plant extracts and quercetin

Tab. 2. Average body weight and feed conversion ratio of broiler chickens

Day 1-32	Additive type					Concentration (g/kg)			SEM	P-value		
	C	RO	OL	PB	Q	0.00	2.50 or 0.25*	5.00 or 0.50*		Additive	Conc.	Additive × Conc.
Weight gain (g)												
	1542	1439	1506	1483	1556	1542[a]	1461[b]	1531[a]	17.058	0.101	0.039	0.102
Feed conversion ratio (kg feed/kg BW gain)												
	1.293[a]	1.428[b]	1.311	1.389	1.312	1.293	1.390	1.330	0.019	0.041	0.076	0.032

Explanation: [a, b] – means in the row with different superscripts differ significantly at $P < 0.05$; *concentration of quercetin

BWG compared to the control group and chickens treated with avilamycin. Šperňáková et al. (32) also noted that the inclusion of rosemary extract (40 ppm) in chicken diet slightly improved performance parameters ($P > 0.05$). Unfortunately, no literature data were found on the use of OL extract and Q in broilers or other livestock. It should be pointed out that the concentration of the substances analyzed here may have an important influence on the weight gain of broilers fed a diet supplemented with phytogenic extracts (3, 21) and that the ideal analytical conditions are significantly different from those on poultry farms. This was confirmed by Aengwanich et al. (3), who claimed that the addition of 100 mg/kg *Tamarindus indica* extract to feed mixture significantly increased the daily body weight gain of broilers compared to the control group and the birds receiving the polyphenolic supplement at higher concentrations. A significant improvement in daily BWG and feed conversion ($P < 0.05$) in broilers fed with *Forsythia suspensa* extract and maintained under heat stress conditions was reported by Wang et al. (36).

Throughout the experiment, FCR was relatively low, ranging between 1.293 kg and 1.428 kg (Tab. 2). Feed conversion indices were significantly lower ($P < 0.05$) for broilers fed with the control mixture compared to broilers consuming the RO-enriched diet. However, interaction between polyphenolic additives and their concentration in the diets was significant ($P < 0.05$). The OL and PB extracts, as well as Q, had no effect on FCR, regardless of their concentration in the diet (Tab. 2). Our results (except for RO) correspond to those of Barreto et al. (7), who reported that the addition of plant extracts to feed mixtures did not change the feed conversion ratio. On the other hand, an improvement in FCR in broilers treated with grape pomace concentrate ($P < 0.05$) and the absence of a similar effect in chickens fed with grape seed extract was shown by Viveros et al. (35).

The effect of dietary extracts and quercetin on the microbiological status of the broiler's ileum. The microbiological status of the ileum content of broiler chickens fed diets containing the plant extracts and quercetin is presented in Tab. 3 and 4.

Our results indicate that the feeding of broilers with RO, OL and PB extracts and with Q led to a noticeable, but insignificant, reduction in the intestinal populations of microorganisms ($P > 0.05$). This observation is mostly consistent with *in vitro* results published by other authors, who reported antibacterial and antifungal action of the supplements used in this study (Tab. 4). Torras et al. (33) pointed out that polyphenols present in OL extract may be involved in non-specific interactions with proteins in bacteria cell walls, which inactivate enzymes and affect proteins transport leading to cell cycle arrest.

This observation can explain the dramatic decrease in the CFUs of *E. coli* (–93%) and *Lactobacillus* spp. (–84%) in the intestinal contents of chickens fed the commercial

Tab. 3. Effects of polyphenolic additives on ileal microorganism populations (CFU) in broiler chickens

Additive and its concentration (g/kg)	CFU				
	Coliform bacteria	*Escherichia coli*	*Lactobacillus* spp.	Yeast and molds	*Clostridium* spp.
C	4.11×10^4	1.92×10^3	1.74×10^3	8.13×10^2	4.50×10^1
RO	2.42×10^4	1.50×10^3	1.95×10^2	2.20×10^2	2.50×10^1
OL	5.91×10^4	1.33×10^2	2.73×10^2	1.37×10^3	2.17×10^1
PB	1.36×10^4	8.22×10^2	2.57×10^2	8.33×10^1	3.50×10^1
Q	1.60×10^3	4.03×10^2	9.35×10^2	8.33×10^1	4.00×10^1
0.00	4.11×10^4	1.92×10^3	1.74×10^3	8.13×10^2	4.50×10^1
2.50 or 0.25*	3.90×10^4	5.03×10^2	2.18×10^2	7.35×10^2	3.42×10^1
5.00 or 0.50*	1.02×10^4	9.28×10^2	6.12×10^2	1.24×10^2	2.67×10^1
SEM	8.363×10^3	2.204×10^2	1.584×10^2	2.178×10^2	6.071×10^0
P-value					
Additive	0.069	0.196	0.145	0.125	0.725
Conc.	0.068	0.362	0.125	0.172	0.565
Additive × Conc.	0.026	0.766	0.092	0.110	0.058

Explanation: *as in Tab. 2

Tab. 4. Comparison of antimicrobial and antifungal properties of the extracts and quercetin

Microorganisms	Polyphenolic additive	Response (%)	Reference
Coliform bacteria	RO	–41% ↓	(6)
	OL*	+44% ↑	no literature data confirming the results*
	PB	–67% ↓	(33)
	Q	–96% ↓	(15, 1)
Escherichia coli	RO	–22% ↓	(2)
	OL*	–93% ↓	(16, 13, 22)
	PB	–57% ↓	(33, 21)
	Q	–79% ↓	(18)
Lactobacillus spp.	RO	–89% ↓	(34)
	OL*	–84% ↓	(13)
	PB	–85% ↓	no literature data confirming the results**
	Q	–46% ↓	(29)
Yeast and molds	RO	–73% ↓	(16, 34)
	OL*	+69% ↑	no literature data confirming the results**
	PB	–90% ↓	(33)
	Q	–95% ↓	(1)
Clostridium spp.	RO	–44% ↓	(17)
	OL*	–52% ↓	no literature data
	PB	–22% ↓	(3)
	Q	–11% ↓	(1)

Explanations: *addition of olive leave extract to broilers' diet stimulated coliform bacteria growth, but adverse effects were noticed on molds and/or yeast. Other authors report antimicrobial activity against coliform bacteria (22) as well as against *Candida albicans* yeast (16); **the only available literature data (21) shows a stimulating effect of pine bark extract on selected strains of *Lactobacillus* spp. in the chicken's ileum.

OL extract, which was also noted in this study. Ojeda-Sana et al. (19) found that the carnosic acid, an essential component of rosemary extracts, altered the cell membrane potential of *E. faecalis* and *S. aureus*. However, an effective bacteriostatic action of dietary RO was noted only against *Lactobacillus* spp. (–89%) and yeasts/molds (–73%). An antibacterial activity of PB against *Lactobacillus* spp. was also observed, which did not correspond with the results of Park (21). That author found a growth-stimulating effect of PB extract on *Lactobacillus* spp. in the intestinal content of chickens and a significant ($P < 0.01$) decrease in the CFUs of *E. coli*. This discrepancy can be explained by the much lower dosage of the extract used in the broiler diet (70 mg/kg vs. 2.5 and 5.0 g/kg) by that author, which could result in a more selective effect of individual polyphenolic compounds of PB on intestinal microorganisms (e.g. *Lactobacillus* spp.). A similar effect was observed for different concentrations of the olive leave extract (i.e. 0.25% and 0.5%). The addition of 0.25% OL to broiler feed (data non shown) resulted in a noticeable increase in the CFUs of coliform bacteria and molds/yeasts, which is at variance with the *in vitro* results of Pereira et al. (22) and Markin et al. (16), but this effect did not occurred when the concentration of dietary OL was increased to 5.0 g/kg (data non shown).

The addition of quercetin to the diet decreased the CFUs of coliform bacteria, *E. coli*, *Lactobacillus* spp., yeasts and molds, as well as, *Clostridium* spp. in the intestinal content by –96%, –79%, –46%, –95% and –11%, respectively (Tab. 4). A particularly large reduction in the CFUs of these microorganisms in chicken intestinal contents was noted in birds fed with the quercetin supplement at a concentration that was 10-fold lower than that of the other additives. Bernard et al. (8) showed that quercetin may block topoisomerase IV in *E. coli*, which leads to cell death, mainly due to the inhibition of DNA synthesis and the induction of a number of double-strand breaks in the chromosome of the bacteria. The mechanism outlined by the above authors may therefore explain the relatively high efficiency of quercetin against *E. coli* in the present study. Quercetin also has a very strong affinity for synthetic phosphatidylcholine membranes (30), which cause a significant unsealing of the liposomal membrane and leakage of the content (unpublished data). This kind of interaction may destabilize bacterial cell membranes and can also explain the large decrease in the CFUs of the selected microorganisms.

The influence of dietary polyphenols on the gut microflora and growth performance of broiler chickens is not yet fully understood. Still only little is known about the mechanism of antimicrobial action of phytogenic additives and their impact on the feed efficiency ratio or nutrient digestion. A clear tendency to reduce the CFUs of bacteria, molds and yeasts in gut contents indicates that quercetin and extracts of rosemary, olive leaves and pine bark could be highly valuable additives in poultry nutrition. On the other hand, there was no corresponding improvement in the basic production parameters of chickens. Our results gave some evidence for further research in this area allowing to determine the optimal dosage of the polyphenol sources tested in this study and to achieve optimal growth performance and bacteriostatic action in the poultry gut.

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