Use of β-Glucanases and β-1,4-Xylanases to Supplement Diets Containing Alfalfa and Rye for Laying Hens: Effects on Bird Performance and Egg Quality

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Primary Audience: Nutritionists, Researchers, Feed Formulators

SUMMARY

It is well established that the use of alfalfa in diets for monogastric animals is limited by its high fiber content. However, alfalfa is a natural source of xanthophylls and gives poultry products a desirably yellow color. In addition, alfalfa saponins may contribute to reduce the levels of cholesterol in the meat and egg yolk. We have investigated the potential utilization of β-glucanases and β-1,4-xylanases for enhancing the nutritive value of alfalfa for laying hens. An experiment was conducted with 864 ISA Brown layer hens from 40 to 52 wk of age and fed on diets containing rye (19.6%) or rye and alfalfa (15.1%). The results suggested that inclusion of alfalfa in the diets reduced body weights and total egg mass ($P < 0.01$). Dietary supplementation with polysaccharidases was unable to significantly improve the performance of laying hens. However, egg yolks from birds that consumed diets containing alfalfa were more deeply pigmented, presenting an increase in yellowness (b*). In contrast, at the percentage of incorporation tested, inclusion of alfalfa in the diets was unable to lower the levels of cholesterol content in the egg yolk. Taken together the results suggest that exogenous plant cell wall hydrolases are not effective for improving the nutritive value of alfalfa-containing diets for laying hens, although inclusion of small percentages of the dehydrated leguminous meal may directly affect the quality of the generated poultry products.

Key words: β-glucanase, β-1-4-xylanase, alfalfa, rye, laying hen

2006 J. Appl. Poult. Res. 15:256–265

DESCRIPTION OF PROBLEM

Microbial β-glucanases and β-1,4-xylanases are widely used for supplementing poultry diets rich in nonstarch polysaccharides (**NSP**). Soluble arabinoxylans and β-glucans lead to a considerable increase in digesta viscosity [1, 2], thus interfering with the movement of particles and solutes across the intestinal lumen and reducing the access of the repertoire of digestive enzymes to their substrates [3]. Endo-acting polysaccharide hydrolases added to the diets decrease the

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degree of polymerization of the recalcitrant NSP, leading to a considerable reduction in digesta viscosities [4]. In addition, the products of cellulase and xylanase activities are more prone to fermentation by the microbial organisms that colonize the last compartments of the gastrointestinal tract, and more energy is consequently absorbed from the hydrolysis of NSP [5]. Finally, breakdown of plant cell wall polysaccharides improves the access of the digestive biocatalysts to the endosperm contents that were otherwise trapped [6]. The various effects of enzyme supplementation in the digestive process are usually reflected by a considerable improvement of growth and feed conversion rates by poultry [7, 8, 9, 10], although the mechanism of action of feed enzymes is not fully understood.

Dehydrated alfalfa (*Medicago sativa* L.) is high in protein (17.5%) and crude fiber (24.1%) but low in metabolizable energy. Previous studies have investigated the use of alfalfa in laying hen nutrition to improve the degree of pigmentation of the egg yolk [11, 12] and as a general protein concentrate for poultry [13, 14]. However, dehydrated alfalfa meal is usually used at very low levels in poultry nutrition, especially due to its high fiber and low energy contents. It has been previously shown that alfalfa slows the passage rates in the avian gastrointestinal tract [15, 16], which could be advantageous because dietary fiber sources may be fermented at a greater extent by microorganisms [17]. In addition, this fermentation could retain microflora that may function, in part, as a barrier to pathogenic bacteria [18]. Indeed, preliminary studies by Kwon and Kubena [19] have shown that alfalfa limited the colonization and infection with *Salmonella enteritidis* in laying hens.

Dehydrated alfalfa is a good source of hypocholesterolemic compounds such as saponins. Saponins have the capacity to link to cholesterol and steroids, impeding its absorption and consequently reducing cholesterol plasma concentrations [20, 21, 22, 23]. Alfalfa consumption has been shown to decrease the cholesterol content of broiler meat [24] and egg yolk [25]. Guclu et al. [26] observed that the addition of 9% alfalfa meal into laying quail diets reduces serum triglycerides and cholesterol and egg yolk cholesterol without any adverse effect on performance. However, Whitehead et al. [27] observed that in laying hens saponins depressed feed consumption, body weight, egg production, liver lipid concentrations, and plasma triglyceride concentrations without affecting lipid excretion, liver cholesterol, and plasma high density lipoprotein and cholesterol concentrations. In addition, egg yolk color is an important factor for egg marketing in several countries. The visual yolk color perceived by the consumer is the result of the deposition of absorbed carotenoids in the egg yolk, namely its xanthophyll subgroup. Some xanthophylls, such as the luteins and zeaxanthin, are present in several feedstuffs such as alfalfa [28], in which contents may reach 140 mg/kg [29]. Thus, alfalfa meal can be included in chicken rations to enhance the yellow color of the egg yolk, although at limited rates of incorporation. In contrast, cereals, such as wheat, barley, and rye, contain a low level of pigmenting agents, and, as a result, the yolks of eggs from birds fed on these diets have pale yellow color.

To our knowledge, potential beneficial effects have not been established for including plant cell wall hydrolases in diets containing moderate to high levels of alfalfa for laying hens. Cellulases and β-1,4-xylanases could contribute to a significant depolymerization of alfalfa plant cell wall polysaccharides, resulting in a considerable release of energy otherwise not available to the animal. This could enhance the exploitation of the positive effects of alfalfa on the quality of poultry products through increasing the percentages of alfalfa incorporation in poultry diets. The objective of the research reported here was to establish the possibility of using exogenous β-glucanases and β-1,4-xylanases to improve the nutritive value of alfalfa for laying hens. Therefore, the influence of introducing plant cell wall hydrolases in diets containing moderate levels of rye and alfalfa on animal performance and egg quality was assessed.

MATERIALS AND METHODS

Birds, Diets, and Management

One experiment was conducted to determine the effects of β-glucanase and β-1,4-xylanase supplementation on the nutritional value of diets containing alfalfa and rye and fed to laying hens and on the quality of eggs produced. A total of

864 ISA Brown layer hens, 40 wk of age, were randomly distributed into 24 replicates (12 cages with 3 birds/cage was considered a replicate experimental unit) and randomly assigned to each of the 4 experimental diets (6 replicates/treatment). The experiment was developed for a period of 12 wk (40 to 52 wk of age). In the preexperimental period (36 to 42 wk), egg production, egg weight, and initial body weight were recorded for hens fed on a typical corn-soybean meal diet. The hens were selected based on the preexperimental egg production and body weight so that the average performance of the hens in each treatment would be similar at the start of the experiment. Each cage was provided with an individual feeder and 2 automatic pipette drinkers. Water was available ad libitum throughout the experiment. The cages were located in a temperature-controlled room, and the photoperiod during the experiment was fixed at 16 h.

The 4 treatments under analysis consisted of 2 basal diets, containing 19.6% rye (diet R) or 20.0% rye and 15.0% dehydrated alfalfa (diet A), not supplemented or supplemented with a commercial cellulase and xylanase enzyme mixture. The composition of the basal diets, formulated according to the National Research Council specifications [30] for laying hens, is shown in Table 1. The commercial enzyme cocktail resulted from the mixture of the 3 different enzyme additives Roxazyme G [31], Avizyme 1100 [32], and Avizyme 1300 [33], at the incorporation rates of 0.01, 0.1, and 0.3% (wt/wt), respectively. Enzymes were mixed with the other ingredients at the final stages of diet preparation. Diets were distributed ad libitum to the birds and were in mash form.

At wk 40, 44, 48, and 52, feed consumption and individual body weights were recorded. Bird mortality, egg production, and percentages of dirty and cracked eggs were recorded daily. Daily production was determined on a shelled-egg weight basis. Weekly, all eggs collected were weighed, and mean egg weight was determined. Every 28 d, 6 eggs per replica (36 eggs per treatment) were individually weighed and classified by weight classes. At the end of the experiments 6 birds from each treatment, one per replicate, were killed by cervical dislocation, and digesta was collected from the various gastrointestinal compartments. The samples were frozen at −20°C for posterior analysis.

Table 1. Composition and calculated analysis of the basal diets

Ingredient (g/kg of feed)	Diet[1]	
	R, RE	A, AE
Rye	196.0	196.0
Dehydrated alfalfa meal	0.0	151.0
Wheat	4.0	4.0
Corn	463.2	444.7
Corn gluten feed	75.7	0.0
Soybean meal (44%)	40.0	22.6
Soybean meal (48%)	106.3	58.1
Soybean oil	11.0	13.6
Fish meal 72%	15.0	30.0
DL-Methionine	1.0	1.7
Dicalcium phosphate	5.8	3.6
Limestone	73.0	66.2
Vitamin and mineral premix[2]	3.0	3.0
Antioxidant	3.0	3.0
NaCl	3.0	2.5
Estimated composition		
Metabolizable energy (kcal/kg of feed)	2,690	2,650
Lysine (g/kg of feed)	8.4	9.0
Methionine (g/kg of feed)	4.5	4.5
Methionine + cystine (g/kg of feed)	7.5	7.0
Calcium (g/kg of feed)	33.0	32.5
Available phosphorus (g/kg of feed)	3.8	3.3
Linoleic acid (g/kg of feed)	15.0	16.0
Analyzed composition		
Dry matter (g/kg of feed)	901	904
Organic matter (g/kg of DM)	883	875
Crude protein (g/kg of DM)	160	144
Starch (g/kg of DM)	371	355
Crude fat (g/kg of DM)	41	44
NDF (g/kg of DM)	146	183

[1]Experimental diets contained rye (R and RE) or rye and alfalfa (A and AE) and were supplemented (RE and AE) or not supplemented (R and A) with a mixture of commercial β-glucanases and β-1,4-xylanases.
[2]Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 2,100 IU; vitamin E, 30 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0.12 mg; calcium pantothenate, 10 mg; vitamin K_3, 5 mg; thiamin, 1.1 mg; riboflavin, 4.5 mg; vitamin B_6, 2.0 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 50 mg; Cu, 10 mg; Mn, 70 mg; Zn, 50 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; butylated hydroxytoluene (BHT), 150 mg; monensin, 100 ppm.

Analytical Procedures

Analyses for dry matter, ether extract, crude protein, and dietary fiber were performed according to the Association of Official Analytical Chemist [34] methods. Egg density was measured using 10 saline solutions with specific

gravities between 1.062 and 1.098. Eggs, collected on a weekly basis, were broken out, and blood spots were visually observed. Yolk color was measured using a chromameter [35] that recorded the 3 color coordinates [lightness (**L***), redness (**a***), and yellowness (**b***)], according to the 1976 CIE color system [36] (Commission Internationale de l'Eclairage). The tip of the chromameter measuring head was placed flat against the surface of the yolk. Changes in egg yolk color were measured as an indirect indicator of yolk carotenoid deposition. Thickness measurements of the eggshells with the membrane intact were taken with a micrometer to the nearest 0.01 mm at 3 random locations about the equator of each egg. At the end of the experiment (52 wk) 12 eggs per treatment were collected for cholesterol measurement by HPLC. Total cholesterol was extracted from lyophilized egg yolks (dry matter), after saponification with saturated methanolic KOH, according to the procedure of Naeemi et al. [37], except that 3 extractions with cyclohexane were used (recoveries were greater than 94%). Cholesterol was separated and quantified by normal phase HPLC [38], using an HPLC system [39] equipped with an autosampler and diode array detector adjusted at 206 nm, with a solvent (3% isopropanol in *n*-hexane) flow rate of 1 mL/min and injection volumes of 30 μL. Total cholesterol content in egg yolk samples was calculated in duplicate, based on the external standard technique from a standard curve for peak area vs. concentration. Cellulase and xylanase assays were performed using carboxymethylcellulose and oat spelt xylan, respectively, according to the methods described by Fontes et al. [40]. Analysis of cellulase and xylanase activity in the digesta contents recovered from various gastrointestinal compartments was assessed with agar plates, by using the polysaccharides referred above at 0.1% (wt/vol) final concentration, in 10 m*M* Tris HCl (pH 7.0). Activity was detected after 16 h incubation at 37°C through the Congo red assay plate as described by Ponte et al. [41].

Statistical Analyses

Statistical evaluation of data related to bird performance and egg quality was conducted by ANOVA using SAS [42] with a multifactorial design with 2 factors, alfalfa and enzyme. The statistical model included the effects of alfalfa (without vs. with), enzyme (without vs. with), and alfalfa-by-enzyme interaction. Means were separated using the Tukey's test. Mortality and frequency of cracked and dirty eggs, blood spots, and meat spots in open eggs were analyzed with the χ^2 test. Unless otherwise stated, differences were considered significant where $P < 0.05$.

RESULTS AND DISCUSSION

Bird Performance

Inclusion of alfalfa (15.1%) in diets for laying hens significantly decreased body weight at wk 44, 48, and 52 and feed intake in the first 4 wk of the experiment (Table 2) In addition, the presence of the dehydrated leguminous meal in the diet reduced egg production, egg weight, and consequently the obtained egg mass (Table 2). This result was consistent throughout the animal trial. Although the diets containing alfalfa presented a slight reduction in protein content, the data suggest that the observed reduction in body weight and egg mass production results from the incorporation of alfalfa in the diet, which contributes to reducing its nutritive value and leads to the observed impaired performances. Interestingly, supplementation of the diets containing moderate levels of rye and rye and alfalfa with exogenous β-glucanases and β-1,4-xylanases was unable to significantly improve bird performance. It is possible that the inability of polysaccharidases to improve bird performance results from enzyme inhibition or proteolysis in the gastrointestinal tracts of the birds. To analyze this possibility, digesta samples collected from the various gastrointestinal compartments were tested for cellulase and xylanase activity by using the Congo red plate assay. The data, presented in Table 3, demonstrated that considerable levels of exogenous cellulase and xylanase activities were present in the crop, duodenum, and ileum of birds fed on diets supplemented with plant cell wall hydrolases. Under the same conditions, no enzyme was detected in the corresponding compartments of birds fed on the control diets with no enzyme. Interestingly, all birds, whether supplemented or not with exogenous enzymes, displayed high levels of polysaccharidase activity in the cecum. Taken together, the results demonstrate that alfalfa reduces the

Table 2. Performance of laying hens fed on diets with or without alfalfa and subjected to a supplementation with a commercial mixture of β-glucanases and β-1,4-xylanases

Variable and period (wk)	Alfalfa		Enzyme		*F*-test[1]			
	Without	With	Without	With	Alfalfa	Enzyme	Alfalfa × enzyme	SEM
Body weight (g)								
40	2,033[a]	1,993[a]	2,013[a]	2,013[a]	0.093	0.983	0.915	16.28
44	1,997[a]	1,863[b]	1,949[a]	1,911[a]	0.000	0.148	0.669	21.81
48	1,973[a]	1,861[b]	1,933[a]	1,899[a]	0.000	0.307	0.155	23.40
52	1,995[a]	1,801[b]	1,908[a]	1,886[a]	0.000	0.530	0.362	28.10
Feed intake (g/d)								
40 to 44	128.4[a]	117.7[b]	123.5[a]	122.6[a]	0.002	0.767	0.924	1.809
44 to 48	112.1[a]	112.7[a]	116.9[a]	107.9[a]	0.920	0.167	0.723	3.091
48 to 52	109.7[a]	103.7[a]	106.9[a]	106.5[a]	0.382	0.958	0.531	3.221
Egg produced (egg/d)								
40 to 44	0.852[a]	0.765[b]	0.819[a]	0.799[a]	0.001	0.365	0.856	0.014
44 to 48	0.851[a]	0.761[b]	0.818[a]	0.793[a]	0.000	0.212	0.779	0.013
48 to 52	0.781[a]	0.711[b]	0.746[a]	0.745[a]	0.001	0.968	0.548	0.011
Egg weight (g)								
40 to 44	63.2[a]	61.7[b]	62.6[a]	62.3[a]	0.019	0.592	0.633	0.315
44 to 48	64.0[a]	62.9[a]	63.6[a]	63.2[a]	0.066	0.493	0.362	0.294
48 to 52	64.2[a]	63.5[b]	63.8[a]	63.9[a]	0.029	0.777	0.735	0.153
Egg mass (g/d)								
40 to 44	53.9[a]	47.3[b]	51.3[a]	49.9[a]	0.001	0.398	0.820	1.039
44 to 48	54.4[a]	47.8[b]	52.1[a]	50.2[a]	0.000	0.174	0.936	0.959
48 to 52	50.1[a]	45.2[b]	47.6[a]	47.6[a]	0.000	0.993	0.599	0.744
Feed per egg[2]								
40 to 44	2.38[a]	2.50[b]	2.41[a]	2.47[a]	0.022	0.246	0.645	0.026
44 to 48	2.06[a]	2.36[b]	2.25[a]	2.16[a]	0.016	0.453	0.676	0.064
48 to 52	2.20[a]	2.31[a]	2.26[a]	2.25[a]	0.505	0.952	0.531	0.076
Mortality (%)					χ^2 test[3]			
40 to 52	1.6	0.0	0.9	0.7	0.023	1.000		

[a,b]Values within lines and in the same factor without a similar superscript are significantly different ($P < 0.05$).
[1]*F*-test probability.
[2]Feed conversion ratio (feed intake/egg mass).
[3]χ^2 test probability.

nutritive value of diets for laying hens, and enzyme supplementation is unable to improve bird performance, although high levels of enzyme activity were detected in the gastrointestinal tract of birds receiving supplements. It is unlikely that enzyme inhibition or proteolysis limited the function of the exogenous polysaccharidases when the moderate to high levels of cellulase and xylanase activities detected in birds receiving the microbial enzymes are considered.

Lack of response to enzyme supplementation is not completely unexpected considering the high percentages of corn (>44%) and the reduced levels of rye (<20%) and alfalfa in the diets. Previously, Lazaro et al. [43] observed increased egg production and improved feed efficiency when supplementing cereal-based diets (wheat, rye, or barley) for laying hens with polysaccharidases. However, the percentages of incorporation of the antinutritive cereals in the diets under analysis were much greater than those used in the experiment reported here, and the different responses of bird performance against enzyme supplementation may, in part, be explained by this factor. In addition, it is now well established that response of laying hens consuming cereal-based diets to enzyme supplementation is variable, depending on various factors. Some works [44] report no significant effects of polysaccharidase supplementation in rye-based diets, for example. One possible explanation for this observation might be the variable concentrations of NSP, such as arabinoxylans and β-glucans, in cereals originated from different sources. More-

Table 3. Qualitative detection of cellulase and xylanase activity in digesta collected from the gastrointestinal compartments of 24 birds fed on a diet containing alfalfa supplemented or not with exogenous β-glucanases and β-1,4-xylanases

Diet and activity[1]	Qualitative polysaccharidase activity[2]			
	Crop	Duodenum	Ileum	Cecum
Xylanase				
R	–/–/–/–/+/–	–/–/–/–/–/–	–/–/–/–/–/–	++/++/+/++/+/+
RE	–/+/+/+/–/+	+/+/+/+/+/+	+/+/+/+/+/+	+/++/+/++/++/+
A	–/–/–/–/–/–	–/–/–/–/–/–	–/+/–/+/–/–	+/++/+/++/+/++
AE	+/+/+/–/+/+	+/+/+/+/+/+	–/+/+/+/–/+	++/++/+/++/+/+
Cellulase				
R	–/–/–/–/+/–	–/–/–/–/–/–	–/–/–/–/–/–	++/++/+/++/+/+
RE	–/+/+/+/+/+	+/+/+/+/+/+	+/+/+/+/+/+	+/++/+/++/++/+
A	–/–/–/–/–/–	–/–/–/–/–/–	–/+/–/+/–/–	+/++/+/++/+/++
AE	+/+/+/–/+/+	+/+/+/+/+/+	–/+/+/+/–/+	++/++/+/++/+/+

[1]Basal diets contained rye (R) or rye and alfalfa (A) and were supplemented with a commercial polysaccharidase mixture (RE and AE). Celulase and xylanase activity were detected in situ using a plate assay system as described in the Materials and Methods. In each row, results are depicted for 6 birds per treatment, separated by the symbol /.
[2]Symbols refer to no (–), medium (+) or high (++) cellulase or xylanase activity.

over, it is also possible that effects of enzyme supplementation in poultry nutrition are less evident in older birds, which is the case when exploiting laying hens for egg production [45].

Previously, we had anticipated that the introduction of cellulases and xylanases in diets containing alfalfa for laying hens could help to improve the levels of reducing sugars released in the gastrointestinal tract and, therefore, allow a greater rate of incorporation of this ingredient. The data presented here suggest that exogenous enzymes were unable to reduce the antinutritive properties associated with the incorporation of alfalfa in poultry diets. A similar effect has been reported for diets containing alfalfa and fed to broiler chicks [46]. It is possible that the inherent complexity of alfalfa plant cell wall affects the function of β-glucanases and β-1,4-xylanases acting on more recalcitrant and complex polysaccharides when compared with the usually targeted arabinoxylans and β-glucans in cereal-based diets. Indeed, cellulases and xylanases that reduce the detrimental effects associated with the intake of wheat, rye, and barley by poultry act on soluble carbohydrates, which are much more prone to hydrolysis when compared with the complex and insoluble polysaccharides that are the bulk of the plant cell wall. Indeed, in cereal-based diets, the improvement of animal performance does not depend on the release of reducing sugars but rather on the reduction of the viscosity of the intestinal contents [4, 47]. However, alfalfa has a high fiber content (457 g/kg), the bulk of which is cellulose (48% NSP) with additional high levels of uronic acids and lignin (128 g/kg) [48]. Therefore, the hydrolysis of these complex carbohydrates might require a highly complex repertoire of enzymes acting in synergy. In addition, hydrolysis of the insoluble plant cell wall components may be dependent on a longer incubation period, which is effectively not viable considering the rapid digesta passage rate through the poultry gastrointestinal tract. The high rate of ingesta passage in chickens may reduce the hydrolytic capacity of exogenous polysaccharidases, which act at considerable slow rates due to the recalcitrance of their substrates. Finally, although in the experiment reported here the feed enzymes were detected in the digesta, it is possible that the enzyme mixture we used was not the most appropriated for the targeted substrates present in alfalfa. Under these circumstances, the most appropriate enzyme mixtures to degrade alfalfa plant cell wall polysaccharides remain to be identified.

Interestingly, reduced mortality was observed in the experimental treatments with alfalfa. It has been previously shown that alfalfa slows the passage rates in the avian gastrointestinal tract and increases the proliferation of indigenous microorganisms in the chicken gastrointestinal tract [15, 16, 17]. This property could have an antagonistic effect over pathogen colonization [18] as observed by Kwon and Kubena [19]

Table 4. Effects of alfalfa intake and β-glucanase and β-1,4-xylanase supplementation on egg weight classes produced by laying hens, wk 40 to 52

Period (wk)	Treatment	Egg weight class in g (%)						χ^2 test[1]
		>75	70–75	65–70	60–65	55–60	50–55	
	Alfalfa							
40 to 44	Without	2.8	5.6	25.0	51.4	13.9	1.4	0.009
	With	1.4	4.2	11.1	40.3	34.7	8.3	
44 to 48	Without	0	0.0	20.8	48.6	26.4	4.2	0.260
	With	0	4.2	12.5	45.8	30.6	6.9	
48 to 52	Without	2.8	9.7	18.1	51.4	15.3	2.8	0.410
	With	1.4	5.6	16.7	43.1	29.2	4.2	
	Enzyme							
40 to 44	Without	2.8	5.6	19.4	47.2	22.2	2.8	0.816
	With	1.4	4.2	16.7	44.4	26.4	6.9	
44 to 48	Without	0	1.4	15.3	50.0	29.2	4.2	0.868
	With	0	2.8	18.1	44.4	27.8	6.9	
48 to 52	Without	1.4	6.9	18.1	48.6	19.4	5.6	0.727
	With	2.8	8.3	16.7	45.8	25.0	1.4	

[1]χ^2 test probability.

and Seo et al. [49], who demonstrated that incorporating alfalfa in poultry diets significantly reduces colonization by *Salmonella enteritidis.*

Egg Quality

Inclusion of alfalfa in poultry diets is normally associated with an increase in the quality of the obtained products. Herein we show that alfalfa and enzyme supplementation did not affect the frequency of cracked or dirty eggs, shell thickness, or egg specific gravity (data not shown). In addition, the frequency of albumen and yolk spots was not affected by the various diets. However, as expected from the previous discussion, there was an association between alfalfa intake and egg size, especially between wk 40 and 44 (Table 4). Indeed, diets containing alfalfa increased the frequency of eggs in the class sizes of reduced weights and reduced the frequency of eggs in the class sizes between 65 and 70 g and more than 70 g (Table 4). This observation is in accordance with the lower mean weight observed in eggs derived from hens consuming alfalfa.

At the end of the trial (wk 52), eggs from hens on the various treatments were recovered for analysis of yolk color. Results of the colorimetric evaluation of the yolk are presented in Table 5 as the CIELAB values of L*, a*, and b*. Birds fed diets containing alfalfa tend to produce eggs with lower L* scores, although the differences were significant at wk 48 only. Interestingly, addition of alfalfa to the diets led to the production of eggs presenting higher a* and b* values. These observations confirmed that the yolks from eggs obtained with diets that incorporated alfalfa were more deeply pigmented with yellow. In addition, the higher a* values also suggested a more intense development of pink and red tones that are usually appreciated in egg yolks. It is well established that an increase in a* and b* values is due to a great content of carotenoids in egg yolk [50]. In addition, high intakes of the yellow-orange plant pigments, xanthophylls, lead to their deposition in the yolk. Hens fed mashes containing alfalfa meal lay eggs with yellow yolks, whereas those eating wheat or rye, which lack of carotenoids, yield light-colored yolks. It is clear that in diets containing high levels of pigment-rich ingredients, such as the corn-based diets used in this experiment, alfalfa can potentiate additional improvements on the yellow and red tones of the egg yolk. This finding is in accordance with those of Belyiavin and Marangos [29], who suggested that to match yolk color of free-range eggs, layer rations must be formulated with a minimum of 40% yellow corn and 5% dried grass or alfalfa meal. Enzyme supplementation had no significant effects on yolk color. Only at wk 44 did enzyme supplementation significantly improve the a* parameter of egg yolk color (*P*

Table 5. Effect of alfalfa intake and enzyme supplementation on egg yolk color

Age (wk) and color parameter	Alfalfa		Enzyme		*F*-test[1]			
	Without	With	Without	With	Alfalfa	Enzyme	Alfalfa × enzyme	SEM
44								
L*	60.306[a]	59.722[a]	60.252[a]	59.776[a]	0.301	0.398	0.131	0.282
a*	–3.117[b]	–2.015[a]	–2.883[b]	–2.248[a]	0.000	0.002	0.038	0.116
b*	42.755[a]	46.306[b]	44.292[a]	44.769[a]	0.000	0.545	0.199	0.419
48								
L*	61.128[a]	59.733[b]	60.989[a]	59.868[a]	0.005	0.023	0.417	0.258
a*	–2.654[b]	–0.801[a]	–1.887[b]	–1.542[b]	0.000	0.186	0.690	0.151
b*	46.778[a]	48.218[a]	47.833[a]	47.164[a]	0.102	0.445	0.945	0.438
52								
L*	60.697[a]	59.962[a]	60.522[a]	60.137[a]	0.080	0.357	0.717	0.209
a*	–3.554[b]	–2.131[a]	–2.948[b]	–2.737[b]	0.000	0.105	0.382	0.088
b*	43.875[b]	47.242[a]	45.800[b]	45.317[b]	0.000	0.493	0.314	0.376

[a,b]Values within lines and in the same parameter without a similar superscript are significantly different ($P < 0.05$).
[1]*F*-test probability.

< 0.05). These results show that the capacity of enzyme supplementation to release pigments from the diets is limited. These results were less evident than those observed by Çiftci et al. [51], who reported a significant improvement in egg yolk color in response to enzyme supplementation on triticale and wheat–triticale-based diets. However, it is possible that pigment bioavailability, as a result of enzyme supplementation, may depend on the type of ingredients present in poultry diets.

Finally, the incorporation of alfalfa at moderate levels in laying hen diets had no significant effects on the cholesterol content of the egg yolk (Table 6). These results do not agree with those of McNaughton [25], who observed a significant reduction on egg yolk cholesterol by feeding alfalfa meal, although at different levels of incorporation than those used in this experiment. Also, Vargas and Naber [52] studied the effect of increasing dietary fiber in rations of varying nutrient density on egg yolk cholesterol and demonstrated that yolk cholesterol tends to increase when laying pullets consume more than 387 kcal of ME/d or gain more than 100 g in body weight. In the present experiment, laying hens fed diets containing alfalfa lost weight, and the estimated energy intake was reduced from 295 to 275 kcal of ME/d. However, these conditions did not affect egg cholesterol content. Cholesterol can be directly obtained from the diet, or it can be synthesized de novo in the animal cells from 2-carbon acetate groups of acetyl-coenzyme A. The percentage of cholesterol arising from the de novo biosynthesis or from the diet basically depends on the levels of dietary cholesterol, because the de novo pathway is under feedback control by dietary cholesterol. Even when the levels of dietary cholesterol are very low, de novo biosynthesis will enable the production of the cholesterol required to supply the large variety of biological processes in which this molecule is involved. Although alfalfa saponins reduce the levels of serum cholesterol, it is clear that birds may compensate for this effect by stimulating the de novo biosynthetic pathway.

Table 6. Cholesterol contents (mg/g of yolk) of egg yolk produced by laying hens at wk 52 consuming alfalfa and subjected to a supplementation with a commercial mixture of β-glucanases and β-1,4-xylanases

	Alfalfa		Enzyme		*F*-test[1]			
	Without	With	Without	With	Alfalfa	Enzyme	Alfalfa × enzyme	SEM
Cholesterol	6.175	6.158	6.134	6.1199	0.898	0.627	0.129	0.066

[1]*F*-test probability.

Therefore, under these conditions, only a direct action on the cholesterol biosynthetic pathway would enable a more radical alteration on the putative final cholesterol levels in the egg. The products that may contribute to reduction of cholesterol levels in the serum or egg yolk do so via a reduction of absorbed cholesterol while preventing incremental de novo synthesis.

CONCLUSIONS AND APPLICATIONS

1. Inclusion of alfalfa in diets of laying hens reduced bird performance expressed in terms of body weight and egg mass.
2. β-Glucanases and β-1,4-xylanases were not effective in attenuating the antinutritive effects that arise from the incorporation of alfalfa in poultry diets.
3. Alfalfa contributed considerably to the pigmentation of egg yolk, particular with yellow tones, but it was unable to affect, at incorporations of 15%, the levels of yolk cholesterol.

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Acknowledgments

This work was supported by Fundação para a Ciência e a Tecnologia (FCT), grant POCTI/CVT/2004/61162, and through grant SFRH/BD/2004/17969 to P. I. P. Ponte.