

Carotenoid-enriched transgenic corn delivers bioavailable carotenoids to poultry and protects them against coccidiosis

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Summary

Carotenoids are health-promoting organic molecules that act as antioxidants and essential nutrients. We show that chickens raised on a diet enriched with an engineered corn variety containing very high levels of four key carotenoids (β -carotene, lycopene, zeaxanthin and lutein) are healthy and accumulate more bioavailable carotenoids in peripheral tissues, muscle, skin and fat, and more retinol in the liver, than birds fed on standard corn diets (including commercial corn supplemented with colour additives). Birds were challenged with the protozoan parasite *Eimeria tenella* and those on the high-carotenoid diet grew normally, suffered only mild disease symptoms (diarrhoea, footpad dermatitis and digital ulcers) and had lower faecal oocyst counts than birds on the control diet. Our results demonstrate that carotenoid-rich corn maintains poultry health and increases the nutritional value of poultry products without the use of feed additives.

Introduction

The biofortification of staple crops with organic nutrients is an innovative approach for the improvement of nutritional health without artificial fortification or supplements (Christou and Twyman, 2004; Gómez-Galera *et al.*, 2010; Zhu *et al.*, 2013). There has been substantial progress in the biofortification of staple crops with vitamins (Farré *et al.*, 2011), including Golden Rice producing high levels of β -carotene (Paine *et al.*, 2005) and Multivitamin Corn simultaneously producing high levels of β -carotene, zeaxanthin, lutein, lycopene, ascorbic acid and folate (Naqvi *et al.*, 2009). However, most early-stage research focuses on the accumulation of nutrients *in planta* and not on the fate of those nutrients after consumption, even though this is a key aspect of the metabolic process. It is important to recognize that the bioavailability of nutrients in staple crops provides a more accurate indicator of nutritional quality than the nutrient content alone (Hirschi, 2008; Sanahuja *et al.*, 2013).

Carotenoids act as antioxidants but some are also vitamins with essential biological functions (Bai *et al.*, 2011; Farré *et al.*, 2010; Liu *et al.*, 2012; Lucas *et al.*, 2014; Surai, 2002; Swiatkiewicz and Arczewska-Włosek, 2011). For example, β -carotene and other pro-vitamin A carotenoids are converted into retinol, an essential component of the visual pigment rhodopsin that also helps to maintain epithelial and immune cells, and into retinoic acid, a coregulator of developmental gene expression (Harrison, 2005; Jiali *et al.*, 2012). Lutein and zeaxanthin accumulate to high levels in the retinal macula and are thought to protect the macula and outer segments of the retina from oxidative stress, thus reducing age-related macular degeneration (Mozaffarieh *et al.*, 2003; Rapp *et al.*, 2000; Semba and Dagnelie, 2003). Vitamin A and

carotenoid metabolism in chickens is closely related to the equivalent process in humans, so chickens are also susceptible to vitamin A deficiency with similar symptoms (Subcommittee on Poultry Nutrition, 1994; Pretorius and Schönfeldt, 2013).

The typical corn-/soybean-based commercial poultry diet does not supply sufficient carotenoids to produce the golden skin preferred by consumers and does not confer additional health benefits such as enhanced protective immunity, which has been observed in greenfinches, gull chicks and other passerines with carotenoid-rich diets (Figueroa *et al.*, 2014; Lucas *et al.*, 2014; Sepp *et al.*, 2011). Natural or synthetic pigments such as canthaxanthin are therefore added to the feed, but this increases the production costs (Castañeda *et al.*, 2005). Plants and herbal products rich in carotenoids have recently been tested for their ability to prevent coccidiosis (Arczewska-Włosek and Świątkiewicz, 2012; Dragan, 2014). This is an economically important poultry disease caused by protozoan parasites of the genus *Eimeria*, which is currently controlled using drugs and vaccines (Leon *et al.*, 2012). Birds succumb to coccidiosis when they ingest sporulated oocysts, which are found in abundance on poultry farms (Drăgan *et al.*, 2014; Massod *et al.*, 2013). The development of alternative control measures to reduce the use of drugs would be beneficial, particularly if such measures could be administered in feed (Abbas *et al.*, 2012; Lee *et al.*, 2010; Wunderlich and Al-quraishy, 2014).

We therefore evaluated commercial-type broilers (Ross 308 males) fed on diets supplemented with four types of corn: (i) the wild-type inbred M37W, which is essentially devoid of carotenoids (control diet), (ii) its engineered derivative M37W-Ph3, which accumulates high levels of β -carotene, lycopene, lutein and zeaxanthin (Zhu *et al.*, 2008), (iii) a standard commercial corn-

based diet with the colour additives normally used in commercial poultry production and (iv) the same standard corn-based diet without additives. We compared animals reared on these diets in terms of general health indicators, the accumulation of bioavailable carotenoids and their ability to withstand challenge with the commercially relevant protozoan parasite *Eimeria tenella* (Hafez, 2008).

Results

Compositional analysis

Standard compositional analysis for humidity, protein, fat, fibre and ash content showed that the diets were substantially equivalent except for carotenoid levels (Table S1). The precise carotenoid profiles in the diets were determined by HPLC showing that the high-carotenoid corn diet was enriched for the four key carotenoids as expected, but also contained higher levels of violaxanthin and β -cryptoxanthin (Table S2).

General health indicators

The high-carotenoid corn diet was initially tested against the control diet with unmodified M37W corn. There were no differences in growth or final body weight (Figure S1) or in the final weight of most organs, the exception being the bursa of Fabricius, a lymphoid gland located on the posterodorsal wall of the cloaca that regresses with sexual maturity but plays an important role in disease resistance. This was heavier in the birds fed on the high-carotenoid diet (Table S3). Histopathological analysis showed no diet-dependent differences in medullary or cortical lymphocyte depletion or vacuolization, and no evidence of bleeding or necrosis.

The CIELAB trichromatic system was used to quantify the lightness, redness and yellowness of prechilled meat and skin tissue from birds in both diet groups, revealing significant differences ($P < 0.001$) as shown in Figure 1. The colour of the skin and meat of birds fed on the high-carotenoid diet was much more intense than in the control group, for example the skin shank redness increased from 0.99 in control birds to 11.1 for those fed on the high-carotenoid diet and the yellowness increased from 21.27 to 45.74 (Table S4). Similar differences were observed in the external cutaneous structures such as the comb and the base of the feathers (Figure 1).

The analysis of haemoglobin levels, haematocrit values and the ratio of different blood cell types on day 35 showed no differences between the diet groups. In each case, the values were similar to standard chicken references (Fudge, 2000; Harrison and Lightfoot, 2006). The haemoglobin levels ranged from 11.3 to 11.7 g/dL in the control and high-carotenoid groups, respectively (Table S5), whereas 7–13 g/dL is considered the normal range. Leucocyte counts, including the specific values for eosinophils, basophils, lymphocytes, monocytes and heterophils, were higher in birds fed on the control diet.

Bioavailability of carotenoids and retinol

We next analysed the levels of different carotenoids in the breast and thigh meat, which are most important from a commercial perspective (Figure 2). The analysis of carotenoid levels in breast meat showed that violaxanthin and β -cryptoxanthin were only present in the birds fed on the high-carotenoid diet and that the levels of lutein, zeaxanthin and β -carotene were significantly higher ($P < 0.001$) in the breast meat of birds fed on the high-carotenoid diet compared to the

control. We found that the levels of lutein, zeaxanthin and β -carotene were 117% (twofold), 2104% (22-fold) and 999% (11-fold) higher in birds reared on the high-carotenoid diet. In contrast, violaxanthin and β -cryptoxanthin were not present in thigh meat, lutein levels were similar in both diet groups, zeaxanthin levels were significantly higher ($P < 0.001$) in the thigh meat of birds fed on the high-carotenoid diet, and there was no β -carotene in the thigh meat suggesting that it had been metabolized into downstream derivatives. The thigh muscles of the birds fed on the high-carotenoid diet contained substantial amounts of oxidation products such as zeaxanthin-5,8-epoxides and β -carotene-5,8-epoxides, suggesting that high levels of zeaxanthin and β -carotene may have accumulated initially but subsequently underwent oxidation.

The analysis of carotenoid levels in breast, thigh and shank skin (Figure S2) showed that lutein levels were higher in the shanks of chickens fed on the high-carotenoid diet but not in the other skin samples, whereas zeaxanthin levels were higher in all three skin samples from chickens fed on the high-carotenoid diet. There was little, if any, β -carotene in any of the skin samples, but as for the meat samples, there were high levels of oxidation products such as zeaxanthin-5,8-epoxides and β -carotene-5,8-epoxides in skin samples from chickens fed on the high-carotenoid diet. We also analysed carotenoid levels in the broiler fat. We found no differences in the lutein content between the diet groups, but much higher levels of zeaxanthin and zeaxanthin-5,8-epoxides in chickens fed on the high-carotenoid diet. There was no β -carotene in the fat, but β -carotene-5,8-epoxides were detected in the fat samples from chickens fed on the high-carotenoid diet (Figure S2). The efficacy of absorption and sequestration in different tissues may differ among the carotenoids. The lutein to zeaxanthin ratio is approximately 3 : 1 in the control feed and 1 : 3 in the high-carotenoid diet (Table S2), and these ratios were approximately maintained in the chicken tissues. The observed differences may reflect the differential absorption, transport or delivery of carotenoids to specific tissues, which may favour the accumulation of zeaxanthin over lutein (Figure S2). The carotenoid composition of the external cutaneous structures also resembled that of the feed except for the absence of α -cryptoxanthin and β -cryptoxanthin in the combs, and β -carotene in the combs and the base of the feathers (Figure S3).

Serum and liver carotenoids and derivatives were compared across all four diet groups. Retinol, zeaxanthin, lutein, α -cryptoxanthin and β -cryptoxanthin were present in the serum of chickens fed on the high-carotenoid diet. Zeaxanthin and retinol were the major components (2.39 and 0.74 $\mu\text{g/ml}$, respectively) and were present at much higher levels than in birds fed the control diet (0.11 and 0.41 $\mu\text{g/ml}$, respectively). The serum from birds fed on standard corn diets with and without colour additives contained 0.86 and 0.92 $\mu\text{g/ml}$ retinol, respectively (Table S6). The serum of animals fed on the high-carotenoid diet was visibly more pigmented than the serum of control animals (Figure 1). Birds reared on the high-carotenoid diet accumulated the highest levels of liver retinol (814 $\mu\text{g/g}$ freeze-dried tissue) compared to the control group (471 $\mu\text{g/g}$) and the commercial diets with and without colour additives, that is 573 and 531 $\mu\text{g/g}$, respectively (Figure 3). The total provitamin A carotenoid content was 6.17 $\mu\text{g/g}$ in the high-carotenoid corn diet, 0.32 $\mu\text{g/g}$ in the control and 0.67 and 0.40 $\mu\text{g/g}$ in the standard commercial diets with and without colour additives, respectively (Table 1).



Figure 1 Morphological comparison of chickens fed on the control and high-carotenoid corn diets. (a) Heads on day 35, showing carotenoid accumulation in tegument structures such as the beak, crest, eyelids and facial feathers in the high-carotenoid diet group. (b) Dissected thighs showing carotenoid accumulation in the skin and cutaneous structures, in the high-carotenoid diet group. (c) Cutaneous structures of the feet. (d) Fat surrounding the gizzard. (e) Serum samples. (f) Carotenoid accumulation in the cutaneous structures at the base of feathers in the high-carotenoid diet group.

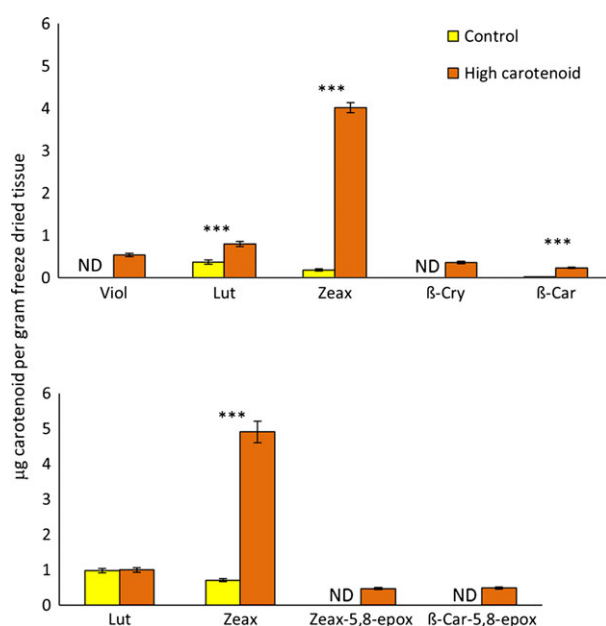


Figure 2 Carotenoid levels in broiler breast (upper chart) and thigh (lower chart) muscle presented as µg/g freeze-dried tissue. Values are means \pm SE for control and high-carotenoid corn ($n = 5$). *** $P < 0.001$. Viol, violaxanthin; Lut, lutein; Zeax, zeaxanthin; β-Cry, β-cryptoxanthin; β-Car, β-carotene; epox, epoxides; ND, nondetectable.

Protection of poultry against *Eimeria tenella*

Following the laboratory-based studies discussed above, a commercial-scale trial was carried out under farm conditions, involving two groups of 24 animals reared on the control and high-carotenoid diets (Figure 4). Having confirmed the nutritional and health-promoting activity of carotenoid-rich corn in a farm

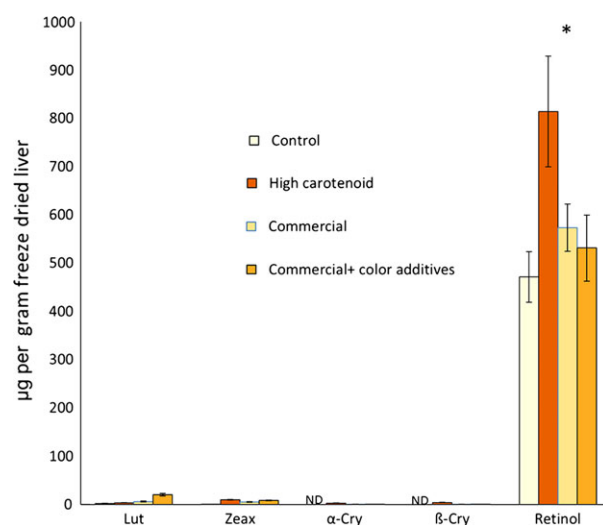


Figure 3 Carotenoid levels in broiler liver. Values are means \pm SE for the four diets ($n = 3$). * $P < 0.05$. Lut, lutein; Zeax, zeaxanthin; α-Cry, α-cryptoxanthin; β-Cry, β-cryptoxanthin; ND, nondetectable.

environment, we carried out an additional feeding trial incorporating a challenge with 3×10^4 sporulated *E. tenella* oocysts (Houghton strain). This is representative of the typical infective dose, which has been estimated as 1×10^4 oocysts, although this is affected by the concurrent use of drugs and vaccines (Drăgan et al., 2014; You, 2014).

We allocated 56 one-day-old Ross 308 males into four groups of 14 animals housed in isolated cages. Two groups were fed on the control diet and two on the high-carotenoid diet. One group from each diet category was challenged orally by gavage with an inoculum of *E. tenella* on day 13, and eight birds per group were killed and assessed for caecal intestinal lesions, foot pad dermatitis and digital ulcers on day 19 (6 days postchallenge). The remain-

Table 1 Pro-vitamin A carotenoid levels and retinol in (i) the four diets (control, high-carotenoid, standard commercial and standard commercial plus colour additives), (ii) serum and (iii) livers from animals reared on these diets

Corn of the diet	Pro-vitamin A and Retinol	Feed ($\mu\text{g/g}$)	Serum ($\mu\text{g/mL}$)	Liver ($\mu\text{g/g fd}$)
Control	β -cryptoxanthin	0.25 ± 0.005	nd	nd
	β -carotene	0.07 ± 0.005	nd	nd
	Total pro-vitamin A carotenoids	0.32		
	Retinol	nd	0.41 ± 0.04	471 ± 52
High carotenoid	β -cryptoxanthin	3.15 ± 0.37	0.21 ± 0.01	3.93 ± 0.43
	β -carotene	3.02 ± 0.31	traces	nd
	Total pro-vitamin A carotenoids	6.17	0.21	3.93
	Retinol	nd	0.74 ± 0.13	814 ± 115
Commercial (Standard)	β -cryptoxanthin	0.46 ± 0.3	0.055 ± 0.01	0.29 ± 0.05
	β -carotene	0.17 ± 0.01	nd	nd
	Total pro-vitamin A carotenoids	0.63	0.055	0.29
	Retinol	nd	0.86 ± 0.11	573 ± 49
Commercial (Standard) + colour additives	β -cryptoxanthin	0.40 ± 0.3	0.69 ± 0.01	0.3 ± 0.02
	β -carotene	traces	nd	nd
	Total pro-vitamin A	0.40	0.69	0.3
	Retinol	nd	0.92 ± 0.25	531 ± 68

Values are means \pm SE ($n = 3\text{--}5$).

fd, freeze dried; nd, not detected.

**Figure 4** Commercial-scale trial under farm conditions. Groups of animals were reared on the control and experimental diets. Each group comprised 24 animals (in randomized cages of 12 animals each) separated from the other animals with fences.

ing six birds per group were assessed for faecal oocyst counts postchallenge and were then killed and analysed as discussed above on day 22.

Animals in the challenged groups weighed 25% less than their counterparts in the nonchallenged groups 6 days postchallenge, reflecting a 25% lower food intake. Caecal intestinal lesions on day 19 (6 days postchallenge) were assigned a score from 0 to 4 depending on severity. The average scores of the eight animals per group sacrificed on day 19 were 0 in the nonchallenged groups on both of the diets, 2.75 ± 0.41 in the challenged group on the high-carotenoid diet and 3.25 ± 0.16 in the challenged group on the control diet. The number of oocysts per gram of faeces in the six remaining animals was 0 in the nonchallenged groups and high in both challenged groups, but there were significantly fewer oocysts in the faeces of chickens fed on the high-carotenoid diet on days 6 and 9 postchallenge (Table 2).

We found that incidences of footpad dermatitis and digital ulcers were significantly lower in animals fed on the carotenoid-rich diet in both the challenged and nonchallenged groups, suggesting that the high-carotenoid diet protects against lesions in the presence and also in the absence of coccidiosis. Our results show that carotenoid-enriched corn can be used to maintain poultry health and immunity (Surai, 2002), thus reducing footpad dermatitis and ulcer counts on the feet due to the more productive inflammatory responses to counteract lesions and secondary infections (Miljković *et al.*, 2012; Shepherd and Fairchild, 2010) (Figure 5).

Discussion

Several differences in general health indicators were noted when comparing the high-carotenoid and control diets. The higher bursa weight in birds fed on the high-carotenoid diet may reflect their better immunomodulatory response to vaccination, given that birds in both groups were given the standard oral Nobilis Gumboro 228 E (MSD Animal Health, Madison, NJ) 'intermediate plus' live vaccine against infectious bursal disease via drinking water on day 14. Poultry vaccines in the 'hot' and 'intermediate plus' virulence categories have been shown to induce morphological lesions in the lymphoid system that reduce the bursal weight compared to animals administered with 'intermediate' or 'mild' vaccines (Moraes *et al.*, 2004). The ability of the high-carotenoid diet to interact beneficially with vaccination schemes indicates that carotenoid-enhanced corn could be used as a complementary strategy to boost resistance to coccidiosis and increase the efficacy of copresented vaccines against coccidiosis and other diseases, although such positive effects are currently speculations and need to be demonstrated in controlled efficacy studies. The high-carotenoid diet may also have promoted faster follicular repopulation than the control diet, thus reducing initial inflammation and enhancing the immune response (Blount *et al.*, 2003; Chew and Park, 2004). This immunomodulatory hypothesis was tested using the same two diets to feed birds vaccinated with the 'intermediate' vaccine Hipragumboro CH/80 (HIPRA, Girona,

Table 2 *E. tenella* faecal oocyst counts. The number of oocysts per gram of faeces was determined four times for all diet groups on days 6 and 9 after infection (days 19 and 22 in terms of age). The T1 (unchallenged control diet) and T3 (unchallenged high-carotenoid diet) groups were not infected, so no oocysts were detected and the data are omitted. The T2 group (control diet challenged with *E. tenella*) showed much higher oocyst counts than the T4 group (high-carotenoid diet challenged with *E. tenella*) on day 9 postinfection

Days postinfection	Oocysts/g faeces T2 ($\times 1000$)	Oocysts/g faeces T4 ($\times 1000$)
6	132.8 \pm 2.9	87 \pm 1.2*
7	223.2 \pm 8.6	241.8 \pm 3.5
8	29.0 \pm 0.7	32.0 \pm 1.5
9	56.9 \pm 4.3	14.2 \pm 0.9*

Values are means \pm SE ($n = 4$).

Significance of data: *0.05 > P > 0.01.

Spain), and accordingly, we found no difference in bursal weight between the test and control groups (data not shown). A link between carotenoids, oxidative stress and the immune system in gulls has previously been described (Lucas *et al.*, 2014).

Like other animals, chickens cannot synthesize carotenoids *de novo* and must obtain them from their food. The deposition of yellow carotenoids in the skin of domestic chickens is enhanced by a recessive allele at the *BCDO2* locus encoding β -carotene dioxygenase 2, which is the genotype of the animals we used (Eriksson *et al.*, 2008). However, the antioxidant activity of carotenoids is also essential for immunomodulation, resulting in their depletion from the circulation during immune stress periods and leading to universally reduced pigmentation (Hamelin and Altemueller, 2012). Consumer preference for broiler skin colour varies by region, but golden skin colour is desirable because it is associated with good health, hence the typical addition of lutein, zeaxanthin and canthaxanthin supplements (Hamelin and Altemueller, 2012). The yellow skin colour in the experimental animals did not lose its intensity during storage, demonstrating the strong antioxidant activity achieved by consuming the carotenoid-rich diet. Chickens selected for high growth rate have paler flesh than animals with a low growth rate, and their breast muscle contains lutein and zeaxanthin but not β -carotene (Le Bihan-Duval *et al.*, 2011). Our data suggest that both the pale flesh and the low β -carotene content of broiler breast meat could be addressed by feeding the birds on a high-carotenoid corn diet.

The depletion of leucocytes in birds fed on the high-carotenoid diet may reflect the better response to the live vaccine as discussed above, because infectious bursal disease induces the loss of leucocytes and vaccines in the higher virulence categories behave in a similar manner (Oladele *et al.*, 2005). The relative and absolute levels of heterophils in chickens are widely used as health indicators because they are influenced by stress, corticosteroid administration and acute inflammatory processes (Gross and Siegel, 1983; Maxwell, 1993). The number of circulating heterophils in birds fed on the control diet was higher than in those fed on the high-carotenoid diet, and this is generally considered to be a response to inflammation or stress (Fudge, 2000).

Serum carotenoids represent the mobile pool of pigments delivered to peripheral tissues, whereas the primary storage tissue

for carotenoids and retinol is the liver (Jlali *et al.*, 2012; Koutsos *et al.*, 2003). The serum carotenoid levels were higher in birds fed on the engineered corn than in those fed on the commercial diet plus additives, but the retinol levels were similar in birds fed on all four diets (Table 1). Serum carotenoid profiles have been reported in other birds (Mcgraw *et al.*, 2006), and both carotenoid and retinol levels tend to be maintained until storage pools are depleted, so they are not considered reliable indicators of nutritional status (Swayne, 2013). In contrast, the livers of birds fed on the engineered corn contained much higher levels of carotenoids and retinol than the other diet groups, including the supplemented commercial diet, probably reflecting the greater supply of β -carotene and β -cryptoxanthin in the engineered corn. This enhanced level of retinol in the liver, the primary location of stored retinol, appears to be one of the key benefits of the carotenoid-enriched corn diet and is likely to be directly linked to the enhanced protective immunity observed in the challenge studies. The absence of β -carotene in most tissues of birds fed on the engineered corn, coupled with the higher levels of β -carotene oxidation products in skin and muscle and the higher levels of retinol in the liver, suggests that excess β -carotene from the engineered corn diet is metabolized to retinol or oxidized in line with its antioxidant activity. Our results therefore suggest that carotenoids from the engineered corn are more bioavailable than those added to standard commercial corn-based diets and preferentially accumulate in the liver, suggesting that liver retinol levels in birds can be used to evaluate the bioavailability of carotenoids, as previously observed in rats (Furusho *et al.*, 2000). The greater bioavailability of intrinsic carotenoids (in the engineered corn) compared to feeds with carotenoid supplements (colour additives) provides an additional incentive for the development of biofortification strategies to achieve nutritional improvement.

The high-carotenoid diet appeared to delay the *E. tenella* reproductive cycle, supporting earlier studies demonstrating the protective effect of carotenoids on other bird species (Figuerola *et al.*, 2014; Lucas *et al.*, 2014; Sepp *et al.*, 2011) and more recent work in which plants and herbal products rich in carotenoids have been tested directly for their ability to prevent coccidiosis (Arczewska-Wlosek and Świątkiewicz, 2012; Dragan, 2014). The drop in the number of oocysts on day 9 postchallenge most likely reflects the parasite life cycle, which lasts 4–7 days (Harrison and Lightfoot, 2006). Following infection with a large number of oocysts, there is a greater likelihood of cycling and reinfection. The ingestion of a large number of new sporulated oocysts initiates a new wave of oocyst development, and the new generation can also be disseminated through faeces. The high-carotenoid diet appeared to promote resistance against oocysts, and the excretion of massive numbers of oocysts was attenuated or delayed. Footpad dermatitis characterized by inflammation and ulcers on the footpad and toes are common in poor litters and can be caused by chemicals, genetic predisposition, immunosuppressive diseases and poor nutrition (Miljković *et al.*, 2012; Shepherd and Fairchild, 2010).

We conclude that poultry raised on the carotenoid-enriched corn diet were healthy and accumulated bioavailable carotenoids. Although liver and serum retinol levels were high in animals reared on the high-carotenoid diet, only traces of β -carotene were found in the liver and breast muscle. However, high levels of β -carotene and zeaxanthin oxidation products were present in blood, breast, thigh and shank skin and fat, indicating that the carotenoids were involved in antioxidant activity in these tissues,

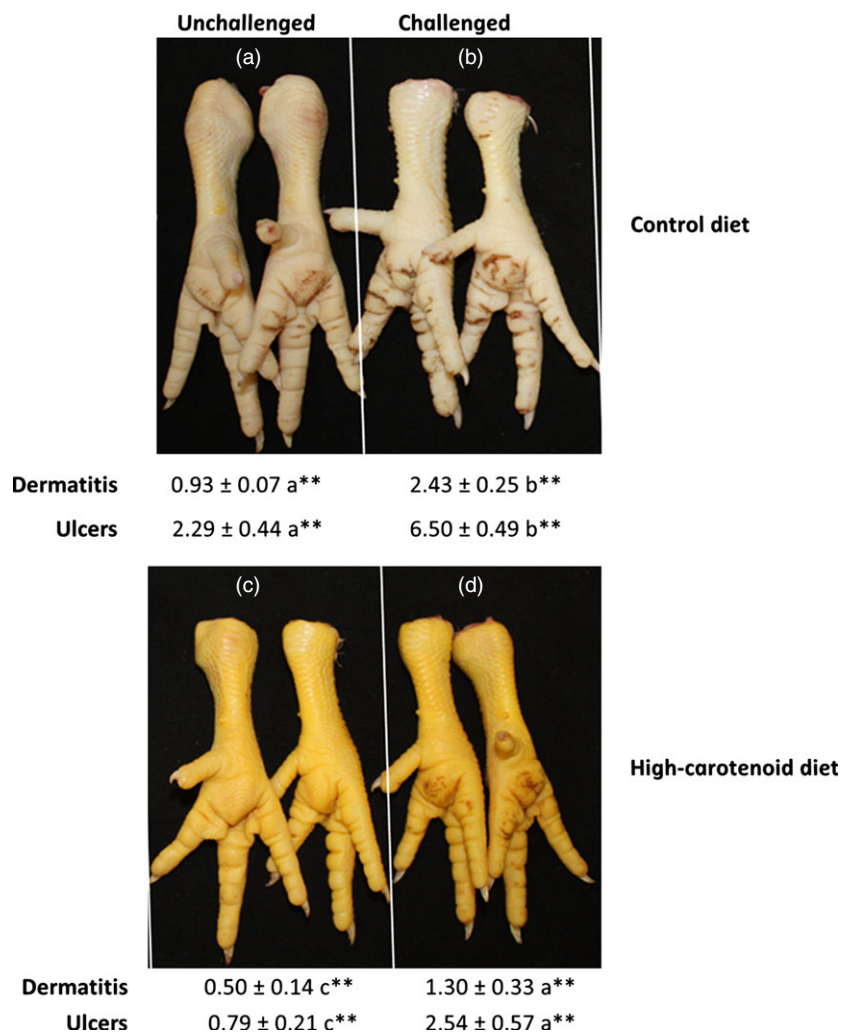


Figure 5 Footpad dermatitis and digital ulcer counts (Welfare Quality®, 2009) on the feet of birds in each treatment category on day 19 or 22 in terms of age. Treatment categories: (a) control diet nonchallenged, (b) control diet challenged with *E. tenella*, (c) high-carotenoid diet nonchallenged and (d) high-carotenoid diet challenged with *E. tenella*. The images show two representative dissected feet from each treatment group at the end of the experiment. Numbers indicate the quantitative values for footpad dermatitis and ulceration. Values are means ± SE ($n = 14$). Values in the same column with a letter in common do not differ significantly (**0.01 > P > 0.001 based on Duncan's test).

protecting the chickens from stress and maintaining the immune system. These health-promoting effects included a reduction in the severity of coccidiosis symptoms concomitant with a delay in the parasite life cycle, reducing the oocyst load in the faeces. Our results demonstrate that carotenoid-rich corn incorporated into commercial poultry diets can maintain poultry health and confer nutritional value to poultry products without the use of expensive feed additives such as canthaxanthin. We can speculate that comparable diets rich in carotenoids may also provide health benefits to other livestock and that based on the similarity between chickens and humans in terms of vitamin A metabolism, carotenoid-enhanced maize could also be used to improve the health of humans, particularly in developing countries where access to nutritious food is limited (Farré *et al.*, 2010, 2011). The levels and composition of carotenoid supplements in commercial diets differ substantially from those delivered through the high-carotenoid corn in our experiments, and the supplements are clearly less bioavailable than the intrinsic carotenoids delivered using the engineered corn variety. To achieve the same levels and composition, commercial diets would need to be supplemented with substantially higher amounts of these beneficial molecules, which is economically unfeasible. Thus, high-carotenoid corn offers an attractive replacement to the standard diet in a commercial setting. Our experiments also pave the way towards feeding trials in humans to investigate whether high-carotenoid corn diets confer similar beneficial effects.

Experimental procedures

Administration of corn-supplemented diets

Four corn-supplemented diets were prepared by mixing ~50 kg of milled corn with a commercial poultry diet and adjusting the other ingredients to maintain the correct nutritional balance (Table S2). The diets were prepared at the Mas de Bover Research Centre (IRTA, Reus, Spain) and formulated according to National Research Council recommendations (Subcommittee on Poultry Nutrition *et al.*, 1994). The high-carotenoid corn diet was based on the M37W-Ph3 carotenoid-enriched corn line (Zhu *et al.*, 2008). The control diet was based on its near isogenic control line M37W. The two additional formulations were commercial corn diets, prepared with or without colour additives (Table S2). The quantity of colour additives was calculated using standard industry methods, taking into account the pigment characteristics of the standard commercial corn used in the diets. Therefore, we added 31 ppm yellow xanthophylls (marigold flower extract, Capsantal EBS-40-NT® itpsa, Barcelona, Spain) and 3 ppm of red xanthophylls (red paprika extract, Capsantal FS-20-NT® itpsa). We measured the humidity, protein, fat, fibre and ash content of the blended corn grains and the formulated diets (Table S1) following Regulation (EC) 152/2009 (27 January 2009) laying down the methods of sampling and analysis for the official control of feed. Control diets were prepared before the carotenoid-enriched diets

to avoid cross-contamination. The diets were administrated in two phases: a starter diet on days 0–8 and a grower diet on days 9–35, as is the norm in commercial poultry production. The birds were fed on a starter diet (52% corn, 36% soybean meal and 7% soybean oil) for 9 days and thereafter on the grower diet (58% corn, 18% soybean meal, 16% soybean hull and 4% soybean oil). We recorded the body weight every 7 days (and on day 9, when we changed to the grower diet).

Broilers, housing, observations and necropsy

Commercial-type broilers (Ross 308 males) were feather-sexed at hatch and obtained from a commercial hatchery, where they were vaccinated against Marek's disease (HVT; Pfizer, New York) and infectious bronchitis (Bronipra; HIPRA). Following a physical examination to confirm the broilers were healthy, they were weighed, identity tagged and randomly allocated into pens (one per treatment) with forced air heaters, individual heat lamps and a cross-house ventilation system that simulates commercial poultry farming practices. After an initial period of constant illumination, the temperature, humidity, lighting and ventilation were monitored and changed according to the age of the birds, following National Research Council guidelines (Committee for the Update of the Guide for the Care and Use of Laboratory Animals *et al.*, 2011). Pens were separated by a wire partition and did not touch other pens to avoid cross-contamination caused by feed spreading. Birds were observed at least three times per day for overall health, behaviour and/or evidence of toxicity. No medication was administered during the feeding trial, but all birds were orally vaccinated via drinking water on day 14 with a live vaccine against infectious bursal disease (Nobilis Gumboro 228 E). Feed and drinking water were provided *ad libitum*.

Experimental design

On day 9, three boilers were allocated to each of eight pens per treatment (24 broilers per treatment) and were started on the grower diet. Body weights and feed weights (the amount of feed offered and the amount remaining) were determined every 7 days and also on day 9 when the feed was changed from starter to grower and the animals were separated. Body weight gain, feed intake and feed efficiency (weight of feed consumed as a proportion of weight gain) were calculated on days 0–35. All birds were humanely euthanized on day 35 and gross necropsy was carried out. Blood samples were collected for biochemical and immunological analysis, and tissue samples were taken for carotenoid analysis. Prechilled samples of breast meat and skin, thigh meat and skin, shank skin, complete combs, liver and abdominal fat were collected and lyophilized for carotenoid analysis. Prechilled whole liver, heart, spleen and bursa of Fabricius were collected and weighed (expressed as g/kg of whole live bird weight). Liver, kidney, spleen, bursa of Fabricius, thymus, tonsils and lung samples were fixed in 4% buffered formaldehyde and embedded in paraffin for sectioning.

Colorimetric analysis

We measured the colour of prechilled carcass, shank, skin and breast and thigh meat with a compact portable spectrophotometer CM-700d (Konica Minolta, Tokyo, Japan). The colour of each sample was measured using the CIELAB trichromatic system as lightness (L^*), redness (a^*) and yellowness (b^*) values. The classical Roche Scale fan was also used to determine shank colour.

Carotenoid analysis

Total carotenoids were extracted from freeze-dried samples in 20 mL methanol containing 6% KOH at 60 °C for 15–20 min. Lipophilic compounds were partitioned into 30% ether in petroleum ether and the upper phase was collected. Total carotenoids were quantified by measuring the absorbance at 450 nm. For HPLC separation, the solvent was evaporated under a stream of nitrogen gas at 37 °C and re-dissolved in 100 µL methanol/dichloromethane (50 : 50), and a 20-µL aliquot was injected immediately. Compounds were separated on a 15-cm Nucleosil C18 3-µL column with an acetonitrile, methanol and 2-propanol mobile phase (85 : 15 : 5 by volume) at 20 °C. Samples were monitored with a Kontron DAD 440 photodiode array detector with online registration of the spectra. All carotenoids were identified by co-chromatography with comparison against authentic reference compounds produced in *Escherichia coli* (Sandmann, 2002). The standards were also used for quantification in combination with appropriate extinction coefficients (Davies, 1976).

E. tenella challenge studies

All procedures involving animals were carried out in accordance with the European Union Guidelines for animal care and under the authorization of the Catalan Government. We randomly allocated 56 one-day-old (Ross 308) male chickens into four equal groups of 15 birds housed in isolated cages. Two of the groups were fed on the high-carotenoid corn diet and two on the control diet, and one group on each diet was challenged with the parasite, giving four treatment groups all together. Thirteen-day-old chicks were challenged with 3×10^4 sporulated *E. tenella* oocysts (Houghton strain, obtained from HIPRA). All birds were weighed individually when they were allocated to the pens and weekly thereafter. Feed efficiency ratio values were calculated weekly as the ratio of feed intake to average weight gain.

Six days after the challenge (19 days after hatching), lesion scores were recorded in all groups by evaluating the caecal intestinal lesions of eight animals per group. Gross lesion scores were assigned numerical values from 0 to 4 as previously described (Johnson and Reid, 1970), where 0 is the normal status with no gross lesions, 1 corresponds to small scattered petechiae (small round spots on the skin), 2 corresponds to numerous petechiae, 3 corresponds to extensive haemorrhage, and 4 corresponds to extensive haemorrhage giving the caecal intestine a dark colour. Dead birds were given a score of 4. Oocyst counts were determined in faecal samples from the remaining six birds per group on days 6 and 9 after infection. Excreta were collected twice on each of the two collection days. Oocyst counts were determined in saturated NaCl solution in McMaster chambers and presented as the number of oocysts per gram of fresh excreta. On day 9 after infection (22 days after hatching), the oocysts in the faeces of the animals were counted individually. The feet of all the animals (eight on day 19 and six on day 22 after hatching) were examined for lesions (footpad dermatitis) as described (Welfare Quality Consortium, 2009). The colour of the shank was also determined as described above.

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Competing financial interests

The authors declare no competing financial interests.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Weekly body weights (g) of broilers fed on the control and high-carotenoid corn-supplemented diets.

Figure S2 Carotenoids in the breast skin (a), thigh skin (b), shank skin (c) and fat (d) of broilers fed on the high-carotenoid and control corn-supplemented diets.

Figure S3 Carotenoids in the comb (a) and feather calamus and rachis (b) of broilers fed on the high-carotenoid and control corn-supplemented diets.

Table S1 Compositional analysis of control corn and high-carotenoid corn and the starter and grower control and experimental diets.

Table S2 Ingredient and carotenoid composition in the control, high-carotenoid, and commercial corn diets with and without color additives (starter and grower diets).

Table S3 Effects of high-carotenoid corn feed on the relative organ weight of broilers (expressed as % relative to whole live animal weight).

Table S4 Color determined using the CIELAB trichromatic system as lightness (L), redness (a) and yellowness (b).

Table S5 Hematology data.

Table S6 Serum biochemical and carotenoid values for the control and high-carotenoid corn groups.