Universitat de Lleida

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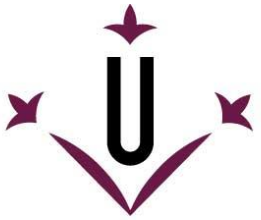
http://hdl.handle.net/10803/405891



Universitat de Lleida

TESI DOCTORAL

High-carotenoid maize as feed and food component: mycotoxin contamination, thermal processing, bioavailability and poultry meat production

Joana Díaz Gómez

Memòria presentada per optar al grau de Doctor
per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia
Agrària i Alimentària

Director
Antonio J. Ramos Girona

Co-directora
Carmina Nogareda Burch

2017

Dissertation presented by Joana Díaz Gómez to obtain the PhD degree from the University of Lleida. This Doctoral Thesis was supervised by Antonio J. Ramos Girona and Carmina Nogareda Burch. The present work was carried out in the Department of Food Technology and the Department of Animal Science, and is included on the Agricultural and Food Science doctoral program. The work is part of the following projects:

- VitaMaize: High quality and safe food through antioxidant fortified maize. Funding entity: RecerCaixa.
- BIO2014-54426-P. Funding entity: Spanish Ministry of Economy and Competitiveness (MINECO).
- BIO2014-54441-P. Funding entity: Spanish Ministry of Economy and Competitiveness (MINECO).

The financing of the PhD candidate was through a project contract (RecerCaixa) and a doctoral grant from the University of Lleida.

This thesis has received a grant for its linguistic revision from the Language Institute of the University of Lleida (2017 call).

Universitat de Lleida
Departament de Tecnologia d'Aliments

Av. Alcalde Rovira Roure, 191
E 25198 LLEIDA (Catalunya)
Tel. +34 973 70 25 21
Fax +34 973 70 25 96
secretaria@tecal.udl.cat
http://www.udl.cat/dept/tecal

El Dr. ANTONIO JAVIER RAMOS GIRONA, Catedrático de Universidad del Departamento de Tecnología de Alimentos de la Universitat de Lleida, y la Dra. CARMINA NOGAREDA BURCH, Profesora Agregada del Departamento de Ciencia Animal de la Universitat de Lleida

HACEN CONSTAR

Que, bajo su dirección, la Sra. Joana Díaz Gómez ha realizado el trabajo de investigación titulado "High-carotenoid maize as feed and food component: mycotoxin contamination, thermal processing, bioavailability and poultry meat production", que presenta para optar al grado de Doctora por la Universitat de Lleida. Considerando que el trabajo realizado constituye tema de Tesis Doctoral, autorizan su exposición y defensa en la Universitat de Lleida.

Y para que así conste, se expide el presente a 14 de junio de 2017.

Dr. Antonio J. Ramos Girona Dra. Carmina Nogareda Burch

Nothing in life is to be feared, it is only to be understood.

Marie Curie

But take your time, think a lot,
why, think of everything you've got
for you will still be here tomorrow
but your dreams may not

Father and Son, Cat Stevens

A mis padres

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

Las cosas podían haber sucedido de cualquier otra manera y, sin embargo, sucedieron así (El Camino, Miguel Delibes). Antes de llegar a *terra ferma*, el camino ha tenido varias etapas previas donde he trabajado con grandes investigadores y compañeros que han contribuido a llegar a esta meta. Esta Tesis Doctoral ha sido posible gracias a la colaboración de varios grupos de investigación pertenecientes al Departamento de Tecnología de Alimentos, al Departamento de Ciencia Animal y al Departamento de Producción Vegetal y Ciencia Forestal de la Universitat de Lleida. ¡Gracias! Gràcies! Thanks!

Quisiera agradecer a mis directores de tesis, Antonio y Carmina, que confiaran en mí para desarrollar este proyecto, que me guiaran y me apoyaran en todo momento. Gracias Antonio por enseñarme el mundo de la micología y por capitanear esta tesis tan multidisciplinar. Moltes gràcies Carmina per estar sempre disponible quan et necessitava, i com no, per tots els detalls que has tingut amb mí i pels sopars de becaris a casa teva, sempre tindré un gran record d'aquells dies.

Un pilar fundamental de esta tesis han sido los directores del proyecto, Paul y Teresa, many thanks for your effort and support to make this work possible. El vostre grup d'investigació és un gran exemple a seguir, estic molt agraïda d'haver sigut part del vostre equip. Many thanks also to Changfu Zhu, Gerhard Sandmann and Richard Twymann for their contribution to my research. Moltes gràcies també à la Núria per sempre ajudar-me amb els temes burocràtics amb la millor somriure.

He tenido muchos tutores adoptivos durante este proyecto, dar las gracias a todos ellos: a Robert Soliva, per estar sempre disposat a donar-me un cop de mà; a Mari Paz, por adentrarme en el mundo del análisis sensorial, y a José Antonio y Eduardo, por sus consejos veterinarios y su implicación en los experimentos con animales. Agradecer también la participación del resto de miembros de los equipos de investigación, en concreto, me gustaría dar las gracias a Vicent y Sonia, por su contribución a mi investigación sobre las micotoxinas, a Jaumes Lloveras y Antonio Michelena, por su trabajo en el campo experimental, y a Ignasi Romagosa por ayudarme con la estadística.

Durante las largas jornadas en el laborarotio he disfrutado de la compañía de técnicos y compañeros siempre dispuestos a comentar el día o a echarte una mano en los momentos críticos, moltes gràcies a tots! Quisiera dar las gracias

a todos aquellos que me ayudaron de un modo u otro en los experimentos con animales (Carmina, José Antonio, Eduardo, Mari Paz, Teresa, Robert S., Montse, Jordi, Robert G., Anna, Magda, los estudiantes de prácticas, etc.), sin vosotros no hubiera sido posible.

He pasado grandes momentos dentro y fuera del laboratorio con mis compañeras de Aluche, Burgos, Palencia, Ushuaia, Zaidín y Zaragoza, ya no podemos seguir resolviendo el mundo en Lleida, pero siempre podemos hacerlo tomando unos pinchos en La Laurel. El destino me había situado en el momento y lugar exacto para que conociera a Laila, Mayka y Sandra en etapas anteriores, pero ha sido la *terra de la boira* la que finalmente nos ha unido. Gracias a todas por acompañarme en esta aventura y por todos los momentos que hemos vivido juntas. También me gustaría recordar a todas mis cuadrillas, como decimos en el norte, las logroñesas, las guaschinas y las nutrias, y a Estefanía, gran investigadora y amiga, cuyo ejemplo me animó a seguir con mi formación científica.

Por último, quisiera agradecer a mis padres y a mi hermana la paciencia y el apoyo durante todos estos años fuera de casa y, sobre todo, el esfuerzo que hicieron para que la pequeña de la casa siguiera formándose. Gracias a mi madre y a mi hermana por comprender mi ausencia en los momentos difíciles de estos últimos años, y a toda la familia y los amigos por el apoyo que prestaron.

La música llega donde las palabras no pueden: *diu que et transporta a un altre planeta, t'inflama el cor amb mil somriures i del cel fa caure espurnes d'or per cada moment que tornes a viure* (Qui n'ha begut, Mishima). Gràcies per sempre ser-hi, encara que sigui a 100 o 400 km de distancia.

ABSTRACT, RESUMEN, RESUM

ABSTRACT

High-carotenoid (HC) maize was genetically engineered to accumulate high levels of carotenoids, including those with vitamin A activity, using as a basis a South African white maize (M37W), which is practically devoid of carotenoids. This biofortified maize was developed to improve the health status of human populations that rely on maize as a staple food crop and cannot access a diverse diet, but it is necessary to evaluate its suitability as feed and food component before fulfilling this humanitarian objective.

During three consecutive harvest seasons (2013, 2014 and 2015), HC maize and its near isogenic line (M37W) were cultivated in an experimental field in Lleida (Catalonia, Northeastern Spain) and both varieties were tested for the prevalence of fungal infection and the presence of fumonisins and aflatoxins. There were only slight differences in fungal infection between both types of maize. *Fusarium* spp. infected most maize kernels; subsequently, fumonisin contamination was found in both maize varieties in all the years of study, but the proportion of contaminated grains was substantially higher in the M37W maize (1.4–fold, 2.4–fold and 2–fold more in the 2013, 2014 and 2015 harvest seasons, respectively). There was no *Aspergillus* spp. infection and, accordingly, there was no contamination with aflatoxins. Maize grains harvested each year also served as raw material to elaborate maize-based feed and maize-derived products.

Since maize is an important cereal source in poultry nutrition, the effect of novel traits on the growth and health of broiler chickens for meat production and thus meat quality needs to be studied. Therefore, two animal feeding trials were performed, including different diets: control (based on M37W maize), HC (based on HC maize) and commercial (based on standard yellow maize supplemented with natural color additives). Generally, productivity and health parameters were similar among chickens fed on the different diets. Chickens fed on the HC diet developed similar pigmentation to those fed on the commercial diet supplemented with color additives, with more intense yellow and red color than chickens fed on the control diet. Thus, HC maize can be a cost-effective alternative to feed supplementation in the poultry industry. The distribution of carotenoids and their derivatives in the chicken was studied in more detail in an animal feeding trial in which the bioavailability of provitamin A (PVA) carotenoids provided as intrinsic components of HC maize compared to the same carotenoids supplied by its

near isogenic line supplemented with synthetic or natural additives was studied. PVA carotenoids from HC maize are bioavailable, at least to the same extent as in synthetic and natural additives, and contribute to liver retinol levels. However, they are not metabolized in the same manner: β-carotene is preferentially converted into retinol in the intestine whereas β-cryptoxanthin accumulates in the liver. Zeaxanthin seems to interfere with β-carotene absorption, since higher liver retinol levels were found in chickens fed on low zeaxanthin diets.

A further aim was to assess the chicken meat quality and sensory shelf life, using as raw material the meat obtained from chickens fed on the control, HC and commercial (plus color additives) diets. Breast and leg quarters from animals fed on the HC diet were equally or better accepted by consumers than meat from the other two diets. Regardless of diet, meat samples were equally accepted after 7–9 days of storage, which could be established as the maximum storage period for this chicken meat accepted by consumers. These results were supported by the analysis of spoilage bacteria (*Pseudomonas* spp. and total aerobic mesophilic bacteria), which increased progressively from 1 to 9 days, and the physical parameters (pH, color and texture), which remained quite similar during storage. Thus, meat from chickens fed on a HC maize diet has a good quality and shelf life as well as a long-lasting golden pigmentation.

Finally, the optimal conditions for preserving carotenoids in HC maize were analyzed, since maize and its derived products have to be cooked before consumption. HC maize-based porridges have been shown not only to preserve the initial carotenoid content, but also to enhance it due to the carotenoid extractability from the food matrix. The carotenoid content was higher when temperatures ≤ 95 °C were combined with short cooking times (10–60 min), but longer times and low pH values can affect it. Regardless of the type of porridge (thin or thick porridges, using unfermented or fermented flours), HC maize-based porridges provided at least 40 μg/g (dry weight) of total carotenoids, and therefore their consumption may contribute to enhancing the dietary status of rural populations who depend on maize as a staple food.

RESUMEN

El maíz HC (del inglés *High-carotenoid*) fue modificado genéticamente para acumular altos niveles de carotenoides, incluyendo aquellos con actividad vitamina A, usando como base un maíz blanco sudafricano (M37W), prácticamente carente de carotenoides. Este maíz biofortificado fue desarrollado para mejorar el estado de salud de las poblaciones que dependen del maíz como alimento básico y no pueden acceder a una dieta diversa, pero es necesario evaluar su idoneidad como pienso y alimento antes de cumplir este objetivo humanitario.

Durante tres cosechas consecutivas (2013, 2014 y 2015), se cultivó el maíz HC y su línea isogénica (M37W) en un campo experimental en Lleida (Cataluña, noreste de España), y se evaluó la prevalencia de infección fúngica y la presencia de fumonisinas y aflatoxinas en ambas variedades. Sólo hubo ligeras diferencias en la infección fúngica entre ambos tipos de maíz. *Fusarium* spp. infectó la mayoría de granos de maíz de ambos tipos, lo que originó que se diera contaminación por fumonisinas en ambas variedades de maíz en todos los años de estudio, aunque la proporción de granos contaminados fue sustancialmente mayor en el maíz M37W (1,4 veces, 2,4 veces y 2 veces más en 2013, 2014 y 2015, respectivamente). No hubo infección por *Aspergillus* spp. y, en consecuencia, no se detectó contaminación con aflatoxinas. El maíz cosechado cada año también sirvió como materia prima para elaborar piensos y productos a base de maíz.

Como el maíz es una importante fuente de cereales en nutrición avícola, es necesario estudiar el efecto de nuevas características en el crecimiento y la salud de los pollos de engorde y, por consiguiente, en la calidad de la carne. Se realizaron dos ensayos de alimentación animal, incluyendo diferentes dietas: control (basada en el maíz M37W), HC (basada en el maíz HC) y comercial (basada en el maíz amarillo estándar suplementado con pigmentos naturales). En general, los parámetros de productividad y salud fueron similares entre los pollos alimentados con las diferentes dietas. Los pollos alimentados con la dieta HC desarrollaron una pigmentación similar a aquellos alimentados con la dieta comercial con pigmentos, con un color amarillo y rojo más intenso que los pollos alimentados con la dieta control. Por lo tanto, el maíz HC puede ser una alternativa rentable a la suplementación de piensos en la industria avícola. La distribución de carotenoides y sus derivados en el pollo se estudió con más detalle en un ensayo de alimentación animal en el que la biodisponibilidad de los

carotenoides provitamina A (PVA), proporcionados como componentes intrínsecos del maíz HC, fue estudiada en relación a los mismos carotenoides suministrados por el maíz M37W suplementado con pigmentos sintéticos o naturales. Los carotenoides PVA del maíz HC son biodisponibles, al menos en la misma medida que en los pigmentos sintéticos y naturales, contribuyendo a los niveles hepáticos de retinol. Sin embargo, no se metabolizan de la misma manera: el β-caroteno se convierte preferentemente en retinol en el intestino mientras que la β-criptoxantina se acumula en el hígado. La zeaxantina parece interferir con la absorción del β-caroteno, ya que se encontraron mayores niveles de retinol hepático en pollos alimentados con dietas bajas en zeaxantina.

La calidad de la carne de pollo y su vida útil sensorial fueron evaluadas utilizando como materia prima la carne obtenida de pollos alimentados con las dietas control, HC y comercial (con pigmentos naturales). Las pechugas y los cuartos traseros de los animales alimentados con la dieta HC fueron igualmente o mejor aceptados por los consumidores que la carne de las otras dos dietas. Independientemente de la dieta, la carne fue igualmente aceptada después de 7–9 días de almacenamiento, lo que podría establecerse como el período máximo aceptado por los consumidores para el almacenamiento de esta carne de pollo. Estos resultados fueron apoyados por el análisis de bacterias alterantes (*Pseudomonas* spp. y bacterias aeróbicas mesófilas), que aumentaron progresivamente del día 1 al 9, y los parámetros físicos (pH, color y textura), que permanecieron similares durante el almacenamiento. Por lo tanto, la carne de pollos alimentados con una dieta basada en el maíz HC tiene una buena calidad y vida útil, así como una pigmentación amarilla-anaranjada de larga duración.

Finalmente, se analizaron las condiciones óptimas para conservar los carotenoides en el maíz HC, ya que el maíz y sus derivados deben ser cocinados antes de su consumo. Los purés de maíz HC han demostrado no sólo conservar el contenido inicial de carotenoides, sino también aumentarlo debido a la extracción de carotenoides de la matriz alimenticia. El contenido de carotenoides se incrementó cuando se combinaron temperaturas ≤ 95 ° C con tiempos de cocción cortos (10–60 min), pero tiempos más largos y valores bajos de pH pueden afectarlo. Independientemente del tipo de puré, (fino o grueso, elaborado con harina fermentada o no), los purés de maíz HC proporcionan al menos 40 μg/g (en peso seco) de carotenoides totales, por lo que su consumo puede contribuir a mejorar la situación alimentaria de las poblaciones rurales que dependen del maíz como alimento básico.

RESUM

El panís HC (de l'anglès *High-carotenoid*) va ser modificat genèticament per acumular alts nivells de carotenoides, incloent aquells amb activitat de la vitamina A, utilitzant com a base un panís blanc sud-africà (M37W), pràcticament sense carotenoides. Aquest panís biofortificat va ser desenvolupat per millorar l'estat de salut de les poblacions que depenen del panís com a aliment bàsic i no poden accedir a una dieta variada, però cal avaluar la seva idoneïtat com a pinso i aliment abans de complir aquest objectiu humanitari.

Durant tres collites consecutives (2013, 2014 i 2015), es van cultivar el panís HC i la seva línia isogènica (M37W) en un camp experimental a Lleida (Catalunya, nord-est d'Espanya), i es van avaluar la prevalença d'infecció fúngica i la presència de fumonisines i aflatoxines en les dues varietats. Només hi va haver lleugeres diferències en la infecció fúngica entre ambdós tipus de panís. *Fusarium* spp. va infectar la majoria de grans de panís, per tant, es va trobar contaminació per fumonisines en les dues varietats en tots els anys d'estudi, però la proporció de grans contaminats va ser substancialment més gran en el panís M37W (1,4 vegades, 2,4 vegades i 2 vegades més en 2013, 2014 i 2015, respectivament). No hi va haver infecció per *Aspergillus* spp., i en conseqüència, no es va detectar contaminació per aflatoxines. El panís collit cada any també va servir com a matèria primera per a elaborar pinsos i productes a base de panís.

Com que el panís és una important font de cereals en nutrició avícola, cal estudiar l'efecte de les noves característiques en el creixement i la salut dels pollastres d'engreix i, per tant, la qualitat de la carn. Es van realitzar dos assajos d'alimentació animal, incloent diferents dietes: control (basada en el panís M37W), HC (basada en el panís HC) i comercial (basada en el panís groc estàndard suplementat amb pigments naturals). En general, els paràmetres de productivitat i salut van ser similars entre els pollastres alimentats amb les diferents dietes. Els pollastres alimentats amb la dieta HC van desenvolupar una pigmentació similar a aquells alimentats amb la dieta comercial amb pigments, amb un color groc i vermell més intens que els pollastres alimentats amb la dieta control. Per tant, el panís HC pot ser una alternativa rendible a la suplementació de pinsos en la indústria avícola. La distribució de carotenoides i els seus derivats en el pollastre es va estudiar amb més detall en un assaig d'alimentació animal en què la biodisponibilitat dels carotenoides provitamina A (PVA) proporcionats com a components

intrínsecs del panís HC va ser estudiada en relació als mateixos carotenoides subministrats pel panís M37W suplementat amb pigments sintètics o naturals. Els carotenoides PVA del panís HC són biodisponibles, almenys en la mateixa mesura que en els pigments sintètics i naturals, contribuint als nivells hepàtics de retinol. No obstant això, no es metabolitzen de la mateixa manera: el β-carotè es converteix preferentment en retinol a l'intestí mentre que la β-criptoxantina s'acumula com a tal al fetge. La zeaxantina sembla interferir amb l'absorció del β-carotè, ja que es van trobar nivells més alts de retinol hepàtic en pollastres alimentats amb dietes baixes en zeaxantina.

La qualitat de la carn de pollastre i la seva vida útil sensorial van ser avaluades utilitzant com a matèria primera la carn obtinguda de pollastres alimentats amb les dietes control, HC i comercial (amb pigments naturals). Els pits i els quarts del darrere dels animals alimentats amb la dieta HC van ser igualment o millor acceptats pels consumidors que la carn de les altres dues dietes. Independentment de la dieta, la carn va ser acceptada per igual després de 7–9 dies d'emmagatzematge, el que podria establir-se com el període màxim d'emmagatzematge per aquesta carn de pollastre acceptat pels consumidors. Aquests resultats van ser recolzats per l'anàlisi de bacteris alterants (*Pseudomonas* spp. i bacteris aeròbics mesòfils), que van augmentar progressivament des del dia 1 al 9, i els paràmetres físics (pH, color i textura), que van romandre similars durant l'emmagatzematge. Per tant, la carn de pollastres alimentats amb una dieta basada en el panís HC té una bona qualitat i vida útil, així com una pigmentació groga-ataronjada de llarga durada.

Finalment, les condicions òptimes per conservar els carotenoides en el panís HC van ser analitzades ja que el panís i els seus derivats han de ser cuinats abans del seu consum. Els purés de panís HC han demostrat no només conservar el contingut inicial de carotenoides, sinó també augmentar-lo a causa de l'extracció de carotenoides de la matriu alimentària. El contingut de carotenoides es va incrementar quan es van combinar temperatures ≤ 95 °C amb temps de cocció curts (10–60 min), però temps més llargs i valors baixos de pH poden afectar-lo. Independentment del tipus de puré, (fi o gruixut, elaborat amb farina fermentada o no), els purés de panís HC proporcionen almenys 40 μg/g (en pes sec) de carotenoides totals, pel que el seu consum pot contribuir a millorar la situació alimentària de les poblacions rurals que depenen del panís com a aliment bàsic.

LIST OF ABBREVIATIONS

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ADG	Average daily gain
AFs	Aflatoxins
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AILP	α-Amylase inhibidor protein
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
a_w	Water activity
β-car	β-Carotene
BCDO1	β-Carotene-15,15'-dioxygenase 1
BCDO2	β-Carotene-9',10'-dioxygenase 2
β-cry	β-Cryptoxanthin
BCW	Black cutworm
BHT	Butylated hydroxytoluene
bp	Base pair
Bt	*Bacillus thuringiensis*
Bw	Body weight
CaSR	Calcium sensing receptor
CD36	Cluster determinant 36

CEW	Corn earworm
cfu	Colony-forming unit
CGIAR	Consultative Group of International Agriculture Research
CIE	Commission Internationale de l'Éclairage – International Commission on Illumination
CPA	Cyclopiazonic acid
CRTISO	Carotenoid isomerase
CRW	Corn rootworm
CSIR	Council for Scientific and Industrial Research
CspB	Bacterial cold shock protein B
CYP97C	Carotene ε-ring hydroxylase
DG18	Dichloran 18% glycerol agar media
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DON	Deoxynivalenol
DRBC	Dichloran rose-bengal chloramphenicol agar media
dw	Dry weight basis
EC	European Commission
ECB	European corn borer
EcfolE	Bacterial GTP cyclohydrolase 1
EDTA	Ethylenediaminetetraacetic acid
em	Emission
EFSA	European Food Safety Authority

EP	European Parliament
EPA	Environmental Protection Agency of the United States
EU	European Union
exc	Excitation
FAO	Food and Agricultural Organization of the United Nations
FAOSTAT	Food and Agricultural Organization of the United Nations Statistics Division
FAW	Fall armyworm
FBs	Fumonisins
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FCR	Feed conversion ratio
fd	Freeze-dried
FDA	Food and Drug Administration of the United States
Fig	Figure
GE	Genetically engineered
GGPP	Geranylgeranyl diphosphate
GGPPS	Geranylgeranyl diphosphate synthase
GM	Genetically modified
GMO	Genetically modified organism
GTP	Guanosine triphosphate
HC	High-carotenoid
HPLC	High-performance liquid chromatography

HSD	Honest significant difference
HYDB	β-Carotene hydroxylase
IARC	International Agency for Research on Cancer
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
ISO	International Organization for Standardization
IU	International units
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of detection
lut	Lutein
LYCB	Lycopene β-cyclase
LYCE	Lycopene ε-cyclase
mc	Moisture content
MCRW	Mexican corn rootworm
MDG	Millenium development goal
MON	Moniliformin
mRNA	Messenger ribonucleic acid
MT	Megatonne
NCRW	Northern corn rootworm
nd	Not detected
ne	Not evaluated
NIV	Nivalenol
NRC	National Research Council

OPA	Ortho-phthalaldehyde
Osdhar	Rice dehydroascorbate reductase
OTA	Ochratoxin A
PacrtI	*Pantoea ananatis* phytoene desaturase
PAT	Patulin
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDS	Phytoene desaturase
Pse	*Pseudomonas* spp.
PSY	Phytoene synthase
PVA	Provitamin A
R	Refrigerated
R	Reproductive stages
RE	Retinol equivalents
RAE	Retinol activity equivalents
RASFF	Rapid Alert System for Food and Feed
RDA	Recommended Dietary Allowance
SCOOP	Scientific Cooperation on Questions Relating to Food
SCN	Standing Committee on Nutrition
SDS	Sodium dodecyl sulfate
SR-BI	Scavenger receptor class B type I

SCARB1	Scavenger receptor class B type I
SWCB	Southwestern corn borer
T	Thawed
TAMB	Total aerobic mesophilic bacteria
TCC	Total carotenoid content
TNAU	Tamil Nadu Agricultural University
UK	United Kingdom
UN	United Nations
US/USA	United States of America
USDA	United States Department of Agriculture
V	Vegetative stages
VAD	Vitamin A deficiency
VE	Emergenge
VT	Tasseling
WB	Warner-Braztler
WBC	Western bean cutworm
WCRW	Western corn rootworm
WHO	World Health Organization
ZDS	ζ-Carotene desaturase
zea	Zeaxanthin
ZEA	Zearalenone
ZISO	ζ-Carotene isomerase
Zmpsy	*Zea mays* phytoene synthase 1

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GENERAL INTRODUCTION

1. Micronutrient malnutrition

Deficiencies in essential vitamins and minerals (micronutrients), so-called hidden hunger, are estimated to affect 2 billion people worldwide, especially women and preschool-age children, due to reproduction and growth demands, respectively. As the term hidden hunger indicates, this form of undernutrition is not readily visible in those affected by it (Muthayya *et al.*, 2013; Nuss and Tanumihardjo, 2010). Iron, iodine, folate, vitamin A, and zinc deficiencies are the most widespread micronutrient deficiencies, and often occur simultaneously, although iodine deficiency is region-specific and is not necessarily related to countries with high hidden hunger (Bailey *et al.*, 2015). Sub-Saharan African countries, as well as India and Afghanistan, have the highest level of hidden hunger, with a high prevalence of stunting, iron deficiency anemia, and vitamin A deficiency (VAD). Micronutrient deficiencies not only have a negative impact on health, but also affect the economic development of those countries (Muthayya *et al.*, 2013).

1.1. Vitamin A

Vitamin A is an essential nutrient that plays several roles in the human body, including normal functioning of the visual system, growth and development, maintenance of epithelial cellular integrity, immune function, and reproduction (UN-SCN, 2004). In many developing countries, VAD is a public health problem that can lead to disorders, such as xerophthalmia (the leading preventable cause of blindness in children), anemia, and weakened host resistance to infection, which can increase the severity of infectious diseases and the risk of death (WHO, 2009). Globally, 5.2 million preschool-age children and 9.8 million pregnant women are estimated to have night blindness, whereas 190 million preschool-age children and 19.1 million pregnant women are estimated to have subclinical VAD, defined as serum retinol concentration less than 0.70 μmol/L (< 20 μg/dL) (Fig. 1) (Black *et al.*, 2008; WHO, 2009).

The main underlying cause of VAD is a diet chronically insufficient in vitamin A, as occurs in poor societies, especially in lower income countries, where the diet is based predominantly on staple crops, such as rice, wheat and maize, whose endosperm lacks sufficient amounts of vitamins and minerals (Gómez-Galera *et al.*, 2010; WHO, 2009). In the human diet, vitamin A comes from animal sources as retinyl esters (mainly retinyl palmitate) or from provitamin A (PVA) carotenoids (e.g. α-carotene, β-

cryptoxanthin and β-carotene) found in plant sources. Other esters (oleate, stearate, myristate) and retinol also contribute to the dietary vitamin A intake (Majchrzak *et al.*, 2006). Retinyl esters from meat and dairy sources are digested to free fatty acids and retinol before uptake by the intestinal mucosal cell, and from there into retinal or retinoic acid. On the other hand, PVA carotenoids are transported into the mucosal cell by scavenger receptor class B type I (SR-BI or SCARB1), where they can be converted into retinal by the enzyme β-carotene 15,15'-dioxygenase 1 (BCDO1), and the retinal is then reduced to retinol by a retinal reductase or is enzymatically used to synthesize retinoic acid (Fig. 2). Generally, about half the dietary PVA carotenoids are converted to retinol and about half are absorbed intact in the small intestine, although this conversion can vary among individuals. β-Carotene is the most efficient and important precursor for vitamin A, since it yields 2 molecules of retinal thanks to its symmetrical structure (Grune *et al.*, 2010; Harrison, 2012).

Figure 1. Prevalence of vitamin A deficiency in children under 5 years (Black *et al.*, 2008).

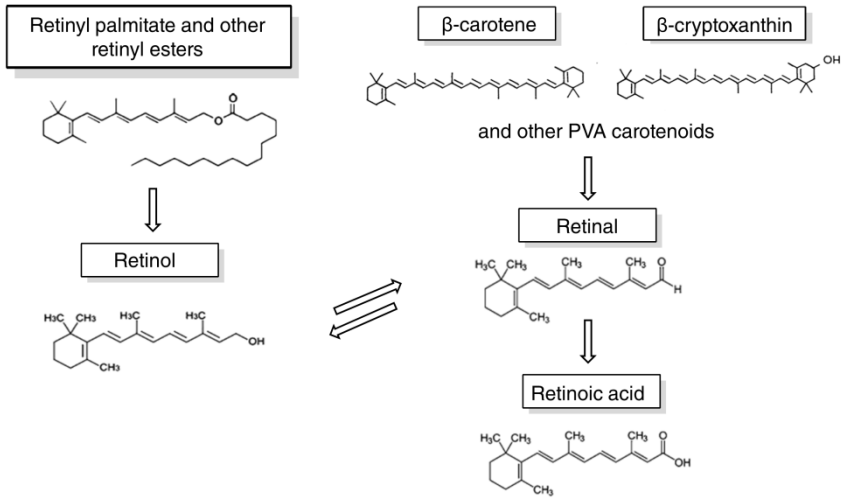

Figure 2. Vitamin A in humans is absorbed as preformed vitamin A or as provitamin A (PVA) carotenoids. Adapted from Bai *et al.* (2011).

Carotenoids are not considered essential nutrients, but it is interesting to note that PVA carotenoids, especially β-carotene, provide more than half of the vitamin A supply in Africa and Asia (Tanumihardjo and Yang, 2009). There are dietary recommendations for vitamin A intake, whereas they are not established for carotenoids. The Recommended Dietary Allowance (RDA) for vitamin A is 900 μg retinol activity equivalents (RAE)/day for men, 700 μg RAE/day for women (which is increased in pregnancy and lactation to 750–770 and 1200–1300 μg RAE/day, respectively) and 300–400 μg RAE/day for children (depending on age). Many factors affect the assimilation of carotenoids from foods, hence the use of conversion factors: 12 μg of β-carotene or 24 μg of other PVA carotenoids are required to obtain 1 μg of RAE (Fig. 3). Food and supplement labels usually indicate vitamin A levels in International Units (IU): one IU of retinol is equivalent to 0.3 μg RAE (Institute of Medicine, 2001).

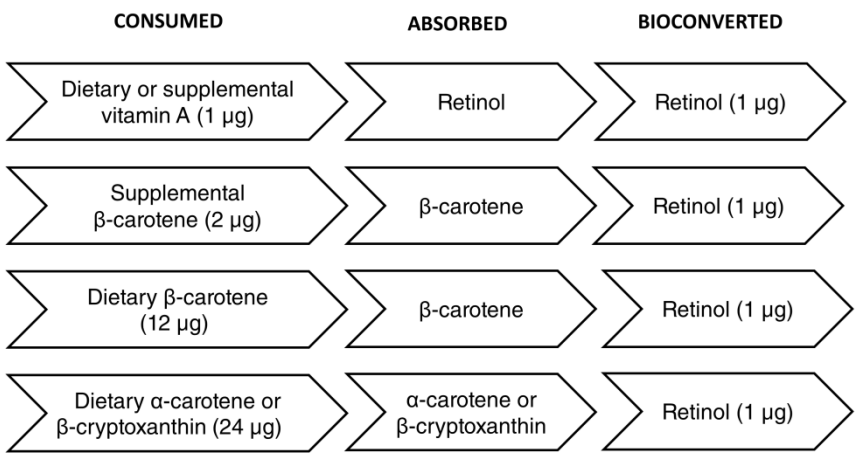

Figure 3. Absorption and bioconversion of ingested provitamin A (PVA) carotenoids to retinol based on conversion factors (retinol activity equivalent). Adapted from Institute of Medicine (2001).

The majority of people in Western societies can easily achieve the RDA for vitamin A, since they have access to foods that are high in preformed vitamin A (e.g. liver, eggs and fortified food) or rich in PVA carotenoids (e.g. fruits and vegetables). Nevertheless, most of the rural population in developing countries are subsistence farmers, who harvest their own staple crops, without having access to a varied diet (Christou and Twyman, 2004). Different strategies have been proposed to deal with VAD including a short-term strategy, based on providing all young children with vitamin A capsules twice a year (commonly known as supplementation programs), a medium-term strategy, based on vitamin A fortification of commonly consumed foods, and a long-term strategy, based on increasing the consumption of foods rich in vitamin A through dietary diversification (Fiedler *et al.*, 2000). Food fortification and supplementation are the most cost-effective strategies to address VAD, but they imply food processing and an adequate supply of vitamin A capsules, respectively, without forgetting an effective distribution network and government support. Most difficult to implement are dietary diversification programs as a long-term solution, as they entail a great investment in agricultural and nutritional programs as well as government funding. Vitamin A supplementation programs, whose cost has been estimated as $130 million per year, have contributed to reduced clinical symptoms such as night blindness. However, subclinical VAD still affects a

high proportion of children in Africa and South Central Asia (Horton *et al.*, 2010; UN-SCN, 2010). Therefore, more efficient interventions such as biofortification will be needed to reduce VAD in those countries. Biofortification of staple crops with organic nutrients could cost-effectively reduce VAD, as exemplified by Golden Rice II (Paine *et al.*, 2005; Ye *et al.*, 2000), Golden Potato (Ducreux *et al.*, 2005) and Carolight® maize (Naqvi *et al.*, 2009; Zhu *et al.*, 2008).

1.2. Carotenoids

Carotenoids are the most widespread group of pigments in nature, with over 600 different carotenoids isolated and structurally characterized, although only about 50 are typically found in the human diet, and only about 20 are present in human blood and tissues, including α-carotene, β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin and α-cryptoxanthin (Fiedor and Burda, 2014). Depending on their chemical structure, they are classified into carotenes (hydrocarbons) and xanthophylls (oxygenated derivatives). Most carotenoids are 40–carbon isoprenoid compounds, biosynthesized by tail-to-tail linkage of two C20 geranylgeranyl diphosphate (GGPP) molecules, from which all individual variations are derived. This C40 hydrocarbon backbone can be modified by cyclization at one or both ends of the molecule, by changes in hydrogenation level and by addition of oxygen-containing functional groups (Britton, 1995; Ishida and Bartley, 2009).

1.2.1. Carotenoid biosynthesis

Carotenoids are biosynthesized by all photosynthetic bacteria, cyanobacteria, algae, higher plants and also by some non-photosynthetic bacteria, fungi, and yeasts. However, humans and most animals are not able to synthesize carotenoids *de novo* and have to obtain them from dietary sources (Namitha and Negi, 2010). There are two exceptions, the red pea aphid (*Acyrthosiphon pisum*) and the two-spotted spider mite (*Tetranychus urticae*), which have acquired the ability to produce carotenoids from fungi by horizontal gene transfer (Altincicek *et al.*, 2012; Moran and Jarvik, 2010).

In plants, carotenoids are synthesized through the condensation of three molecules of isopentenyl diphosphate (IPP) with one molecule of dimethylallyl diphosphate (DMAPP) to form the 20–carbon precursor GGPP, catalyzed by GGPP synthase (GGPPS). Two molecules of GGPP are combined to form the first carotenoid in the biosynthetic pathway, 15-*cis*-phytoene, catalyzed by phytoene synthase (PSY). This intermediate then

undergoes a two-step desaturation reaction in plants to generate 9,15-*cis*-phyfluene and then 9,15,9'-tri-*cis*-ζ-carotene, catalyzed by phytoene desaturase (PDS). This is isomerized by light and/or ζ-carotene isomerase (ZISO) to yield 9,9'-di-*cis*-ζ-carotene, which is converted by ζ-carotene desaturase (ZDS) into 7,9,9'-tri-*cis*-neurosporene and then 7,9,7'9'-tetra-*cis*-lycopene. The final product of desaturation reactions is converted to all-*trans*-lycopene by carotenoid isomerase (CRTISO) in non-green tissue, and by light and chlorophyll (acting as a sensitizer) in green tissue. Nearly all other carotenoids can be derived from lycopene, which can be cyclized at both ends by lycopene β-cyclase (LYCB) to generate the β-ionone end groups of β-carotene, or can be cyclized at one end by lycopene ε-cyclase (LYCE) and at the other by LYCB to introduce the non-identical ε- and β-ionone end groups of α-carotene. Both these molecules can be oxygenated to form xantophylls (e.g. β-cryptoxanthin, zeaxanthin or lutein) by carotene hydroxylases, such as β-carotene hydroxylase (HYBD) (Berman *et al.*, 2015; Farré *et al.*, 2011). These reactions are shown schematically in Figure 4.

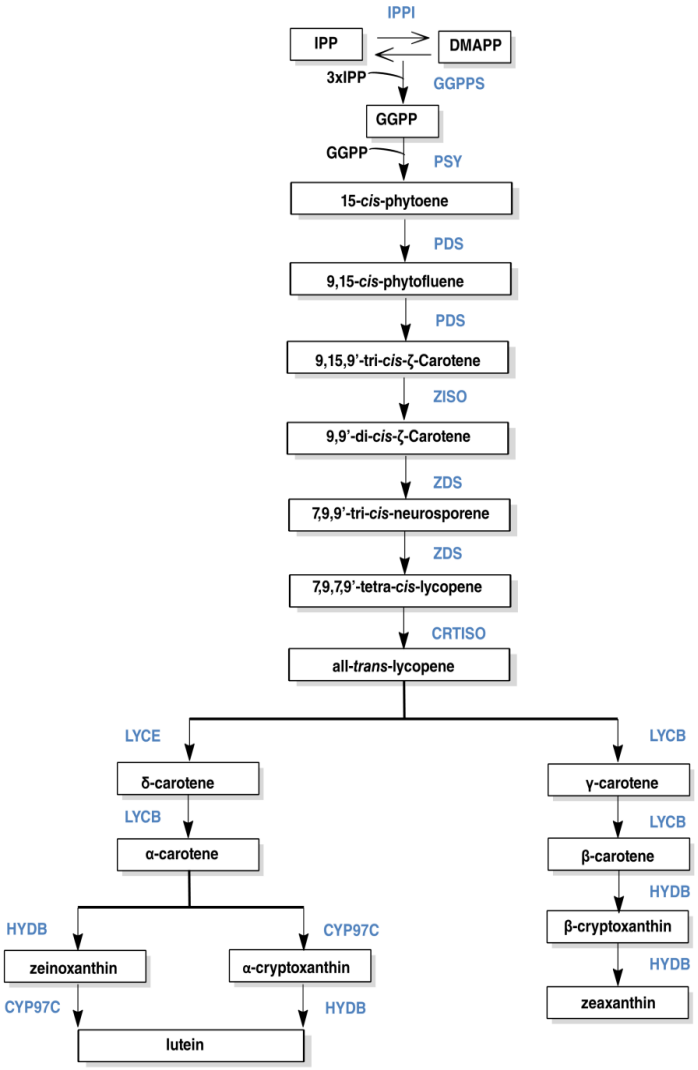

Figure 4. Carotenoid biosynthesis in maize endosperm. Abbreviations: IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene hydroxylase. Adapted from Berman *et al.* (2015) and Farré *et al.* (2011).

1.2.2. Carotenoids: sources and health effects

Some carotenoids exhibit PVA activity (e.g. α-carotene, β-cryptoxanthin and β-carotene), while others act as antioxidants. Apart from its contribution to vitamin A intake, β-carotene, mainly found in carrots, squash, sweet potato, and green leafy vegetables, is an efficient singlet oxygen quencher and also scavenges reactive oxygen species, especially peroxyl radicals. However, several *in vitro* studies have pointed out its prooxidant activity under oxidizing conditions in high levels of oxygen (Grune *et al.*, 2010; Tanumihardjo and Yang, 2009). β-Cryptoxanthin, which also has PVA activity, is primarily found in oranges, tangerines, papaya and peaches, and has been associated with lower rates of lung cancer, whereas high amounts of β-carotene (20–30 mg/day) were related to higher incidence of lung cancer in smokers and asbestos-exposed workers (Krinsky and Johnson, 2005; Tanumihardjo and Yang, 2009). α-Carotene, another PVA carotenoid, is present in high content in carrots and has been associated with lower risk of heart disease and cancer, although few studies have directly examined its role in human health (Tanumihardjo and Yang, 2009).

Most carotenoids efficiently scavenge singlet molecular oxygen and peroxyl radicals, but their antioxidant potential can vary among them. Lycopene, predominantly derived from tomatoes and tomato products, has 2–fold the activity of β-carotene for quenching singlet oxygen. A decreased risk of prostate cancer and cardiovascular disease has been associated with its consumption (Tanumihardjo and Yang, 2009). The structural isomers lutein and zeaxanthin, commonly found in maize, green leafy vegetables and egg yolks, constitute the macular pigment of the human retina, where they protect the photoreceptor cells against free radicals and damaging blue light. Several studies have pointed out their role in protecting against age-related macular degeneration and preventing cataract development (Abdel-Aal *et al.*, 2013).

Fruits and vegetables are good sources of carotenoids that can contribute to human health thanks to their antioxidant properties and/or PVA activity. Nonetheless, their bioaccessibility (i.e. the amount of an ingested nutrient that is released from the food matrix in the gastrointestinal tract) and bioavailability (i.e. the amount of an ingested nutrient that is available for utilization and storage) vary depending on food matrix, dietary components, food processing and storage, among other factors (Carbonell-Capella *et al.*, 2014). Carotenoids can be released from the food matrix, as well as their

promoters and inhibitors, thanks to food processing. For instance, carotenoid bioavailability is enhanced by the presence of fats, whereas it is decreased by soluble fibers, sterols and stanols (Fernández-García *et al.*, 2012; Yonekura and Nagao, 2007). Most strategies developed to address vitamin deficiencies focus on the total amount of nutrient provided rather than nutrient bioavailability. Thus, as many factors affect the efficiency with which nutrients are taken from food, their bioavailability must be taken into account when selecting an appropriate strategy for combating VAD in populations at risk (Sanahuja *et al.*, 2013).

2. Biofortification of staple crops

It is widely acknowledged that biofortification, the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology (WHO, 2016), is an efficient and economical method to improve health in humans and in domestic animals, in the latter case also increasing the value of meat and dairy products. The benefits of added nutrients are well documented, which is why staples such as bread and salt are artificially fortified with minerals and vitamins, and why animal feed is supplemented with additional nutrients. The difference with biofortification is that the plants make or accumulate the necessary nutrients at source and artificial supplements are not necessary (Gómez-Galera *et al.*, 2010). Most of the population in developing countries depends on diets mainly based on staple crops (e.g. wheat, rice or maize) that are poor sources of some macronutrients and many essential micronutrients (DellaPenna, 1999). Biofortification provides a sustainable, cost-effective, long-term strategy for delivering micronutrients to rural populations in developing countries. The three common approaches that are used to increase the nutrient content of food crops are described below.

2.1. Agronomic practices

Agronomic practices are based on the application of mineral fertilizers and the improvement of the solubilization and mobilization of mineral elements in the soil, so-called soil fertilization. However, this strategy can only be used for fortifying plants with mineral elements and not with organic nutrients (e.g. vitamins), which must be synthesized by the plant itself (Carvalho and Vasconcelos, 2013). Fertilization strategies targeted at improvement of mineral content in cereal grains have been partially successful, e.g. zinc concentration has been significantly increased in wheat endosperm, while

iron appears to be difficult in this regard (Clemens, 2014). An alternative method for applying fertilizers in situations where mineral elements are not readily translocated to edible tissues is foliar fertilization, which achieves higher levels of zinc accumulation in maize, rice and wheat than soil fertilization (Carvalho and Vasconcelos, 2013; Joy *et al.*, 2015). The success of these methods depends on the crop species and cultivar, the mineral itself and the soil. Furthermore, it is necessary to apply the fertilizer regularly, which makes this strategy costly, difficult to apply and potentially negative for the environment (Carvalho and Vasconcelos, 2013; Zhu *et al.*, 2007). It must be kept in mind that mineral biofortification is most efficient when cereals are not consumed as flours, because dehulling (which removes the outer layers from the grains) and milling (which reduces the grain size) can affect the mineral bioaccessibility (Raes *et al.*, 2014).

2.2. Conventional breeding

Conventional breeding is based on the identification of parent lines with high vitamin or mineral levels that are crossed over several generations to produce plants that have the desired nutrient and agronomic traits (Rawat *et al.*, 2013). It takes advantage of the plant genetic resources (i.e. the number and quality of crop species and varieties), but it implies considerable efforts to identify genetic variability (Johns and Eyzaguirre, 2007). This strategy is the focus of a biofortification program within the Consultative Group on International Agriculture Research (CGIAR), the HarvestPlus program, which targets a multitude of crops that are a regular part of the staple-based diets of the poor and breeds them to be rich in iron, zinc, and PVA (Pfeiffer and McClafferty, 2007). In 2015, biofortified crops under the HarvestPlus program were released in all eight target countries: Bangladesh (zinc rice), Democratic Republic of Congo (iron beans, vitamin A cassava), India (iron pearl millet), Nigeria (vitamin A cassava, vitamin A maize), Rwanda (iron beans), Uganda (vitamin A sweet potato, iron beans), and Zambia (vitamin A maize) (HarvestPlus, 2017). Alternatively, new traits can be introduced directly into the commercial cultivars by mutagenesis, as exemplified by cereals and legumes with low-phytate content, in which the bioavailability of iron and zinc is increased (Zhu *et al.*, 2007).

2.3. Genetic engineering

According to the definition of the *Codex Alimentarius* (adapted from the *Cartagena Biosafety Protocol* under the Convention on Biological Diversity),

modern biotechnology is defined as the application of: i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles; or ii) fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombinant barriers and that are not techniques used in traditional breeding and selection. On the other hand, a **conventional counterpart** is defined as a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food. In the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts (WHO and FAO, 2009).

Modern biotechnology enables plants to be genetically modified (GM) with novel traits beyond what is possible through conventional breeding (WHO, 2005; WHO and FAO, 2009). Most GM crops cultivated so far have been developed using *Agrobacterium*- and particle bombardment-mediated transformations. As a natural form of genetic engineering, the ubiquitous soil bacterium *Agrobacterium tumefaciens* has the natural ability to transfer a segment of DNA, carried on a specific plasmid, to the plant cell (Zupan and Zambryski, 1995). Other methods were developed to avoid the dependence on bacteria, including particle bombardment, microinjection, electroporation and chemical transfection mediated by polyethylene glycol or calcium phosphate transformation. Among these direct DNA transfer methods particle bombardment, which employs high velocity DNA-coated metal particles to deliver biologically active DNA into plant cells, is the most widely used. This versatile and effective transformation method is not limited by cell type, species or genotype (Altpeter *et al.*, 2005; Christou, 1992).

Depending on the aim of the trait introduced, there are three generations of biotech crops (Fig. 5). The first generation was generated to improve productivity through the modification of agronomic traits; the second generation focused on product quality characteristics, such as more nutritional content; and the third generation aimed to produce pharmaceutical and industrial molecules (Yonekura-Sakakibara and Saito, 2006).

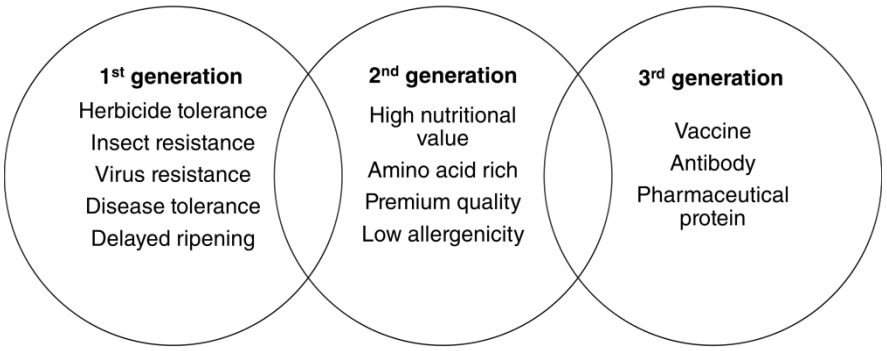

Figure 5. Three generations of biotech crops. Adapted from Yonekura-Sakakibara and Saito (2006).

The first commercial genetically engineered crops were deployed in 1996 (1.7 million hectares in six countries, which have grown to 179.7 million hectares in 28 countries in 2015) (Fig. 6). The major biotech crops planted worldwide are soybean, maize, cotton and canola, with the United States, Brazil, Argentina, India and Canada as the biggest producers. In the European Union (EU), five countries (Spain, Portugal, Czech Republic, Slovakia and Romania) planted 116,870 hectares of Bt (*Bacillus thuringiensis*) maize in 2015, which were reduced 18% from 2014 mainly due to less maize planted. Spain led the production with 107,749 hectares of Bt maize planted (James, 2016). While the first generation of GM plants (e.g. herbicide-tolerant soybean, insect-resistant maize and delayed ripening tomato) was mostly beneficial to producers, the next generations of GM plants are mainly focused on consumer benefits (e.g. vitamin A-biofortified rice and folate-biofortified rice). Accordingly, public opinion is more favorable to these second and third generations of biotech crops, which are still in development, than to the first generation of biotech crops, which is currently in the fields (De Steur *et al.*, 2016; Stewart and McLean, 2005).

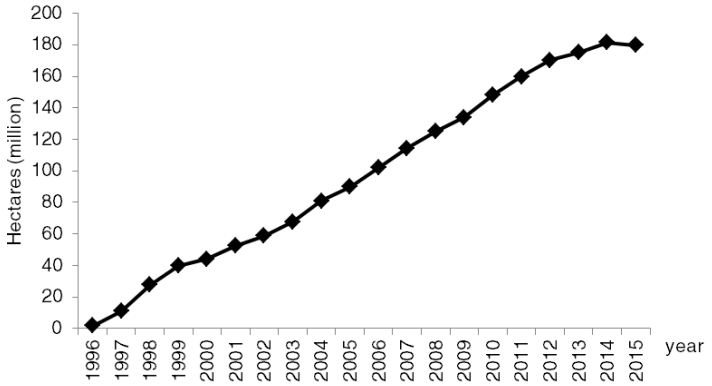

Figure 6. Global area of biotech crops, 1996 to 2015. Adapted from James (2016).

Genetic engineering allows the direct modulation of metabolic pathways for the nutritional enhancement of crops, either by enriching them with novel nutrients or increasing the content of the previously existing nutrients or decreasing undesirable compounds (Kamthan *et al.*, 2016). A novel strategy based on combinatorial genetic transformation opens the door to multiplex-transgene plants, enabling the development of nutritionally completed cereals, which could generate substantial health effects at a relatively low cost (De Steur *et al.*, 2016; Zhu *et al.*, 2008).

3. Maize

Maize (*Zea mays* L.), also known as corn, is believed to have originated in Mesoamerica, particularly in Mexico, 7,000 years ago from a wild grass, probably teosinte. However, this wild grass was transformed into maize by native populations for a better source of food. After the discovery of the American continent by early settlers, maize was introduced into Europe through Spain, and then spread through the warmer climates of the Mediterranean and Northern Europe, and later traders brought maize to Africa and Asia (FAO, 1992; Ranum *et al.*, 2014).

Botanically, maize belongs to the grass family (*Gramineae*) and is a tall annual plant which reproduces via crosspollination, with the female (ear) and male (tassel) flowers in separate places on the plant. The plant development is divided into two physiological stages, vegetative and reproductive (Fig. 7a). In the vegetative stages, which include from emergence (VE) to tasseling

(VT), different tissues develop (e.g. leaves and reproductive organs) and differentiate until the flower structures appear. The reproductive stages begin with the fertilization of the female structures (which will develop into ears and grains) and end with a rapid increase of the kernels' weight. The reproductive stages include: silking (R1), in which any silk is visible outside the husk; blister (R2), in which kernels are white and resemble a blister in shape; milk (R3), in which kernels are yellow on the outside with a milky white inner fluid; dough (R4), in which starch has a dough-like consistency; dent (R5), in which kernels are dented; and physiological maturity (R6), in which all kernels on the ear have reached maximum dry weight with physiological maturity. These mature kernels are harvested for consumption and processing (FAO, 1992; He *et al.*, 2010).

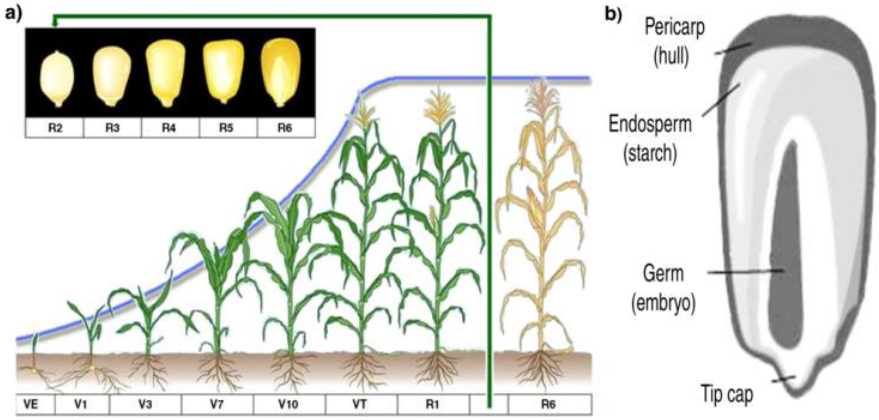

Figure 7. a) Different growth stages of a typical maize plant, including vegetative (V) and reproductive (R) stages. Vegetative stages: VE, emergence; V1, first leaf collar; V3, third leaf collar; V7, seventh leaf collar; V10, tenth leaf collar; VT, tasseling. Reproductive stages: R1, silking; R2, blister; R3, milk; R4, dough; R5, dent; R6, maturity (He *et al.*, 2010). **b)** Structure of the maize kernel.

Each kernel is composed of four different compartments (Fig. 7b): endosperm (mainly starch surrounded by a protein matrix) (83%); embryo or germ (high in polyunsaturated fatty acids, enzymes and nutrients) (11%); pericarp (a seed coat high in fiber) (5%); and tip cap (high in fiber) (1%). The latter, the only structure not covered by the pericarp, is the connection of the kernel to the cob, through which water and nutrients pass during development (Gwirtz and Garcia-Casal, 2014). Maize contains approximately 74% carbohydrates, 9% protein, 5% lipid, supplying an energy density of 365

kcal/100 g and providing many of the B vitamins and essential minerals as well as fiber (7%) (USDA, 2016). However, other important micronutrients are not present (e.g. vitamin B12, vitamin C and the essential amino acids lysine and tryptophan) or are present at low levels (e.g. iron, calcium and folate) (Nuss and Tanumihardjo, 2010; Ranum *et al.*, 2014).

Generally, humans consume maize in different forms such as maize grits, polenta, porridge, maize bread, popcorn and maize flakes, and it can even be used to produce beer. Maize can be processed in the household on a small local scale or on a larger industrial scale into different maize constituents (Fig. 8). The initial processing (e.g. dehulling or degerming and milling) depends on regional preferences, but can result in the loss of most vitamins and minerals, as they are concentrated in the outer layers of the maize grain. Some maize products can be used for commercial trade, while others have to be consumed immediately after production. In both cases, maize products have to be further processed to be palatable and different cooking techniques can be applied according to regional customs. For instance, the grain is usually fermented in African countries, whereas it is nixtamalized (i.e. soaking maize grains in an alkaline solution) in Central America (FAO, 1992; Gwirtz and Garcia-Casal, 2014).

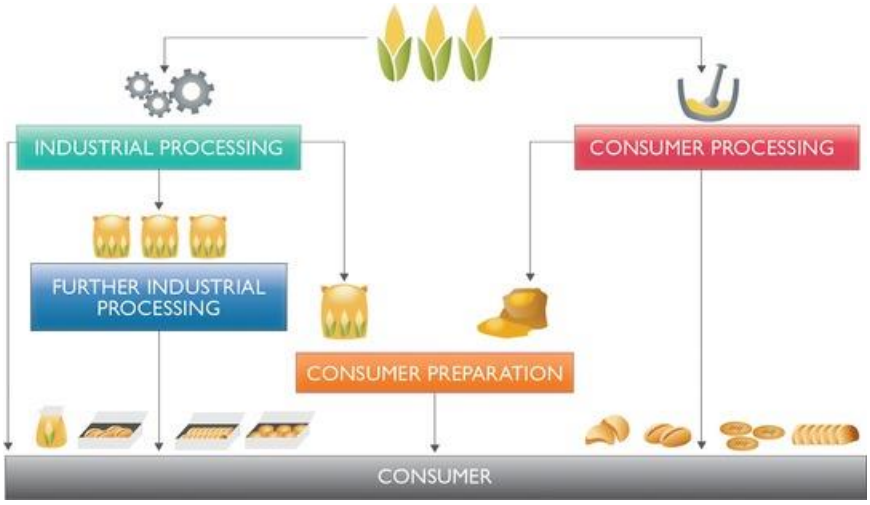

Figure 8. Processing maize from raw material into food products (Gwirtz and Garcia-Casal, 2014).

Maize is the cereal grain most produced worldwide (32%), followed by wheat (31%) and rice (22%), with the United States, China, Brazil, Mexico and

Argentina as the major producers (Table 1). Despite the continuous increase in its production in the last two decades (Fig. 9), its human consumption (12%) is lower compared to wheat (44%) and rice (37%), which can be explained by its use for fuel production or as feed ingredient for livestock (Nuss and Tanumihardjo, 2010; Ranum *et al.*, 2014).

Table 1. Maize production in 2014, by country (FAOSTAT, 2015).

Country	Maize production in 2014 (MT/year)
United States of America	361
China	216
Brazil	80
Mexico	23
Argentina	33
India	24
Ukraine	28
Indonesia	19
France	18
South Africa	14

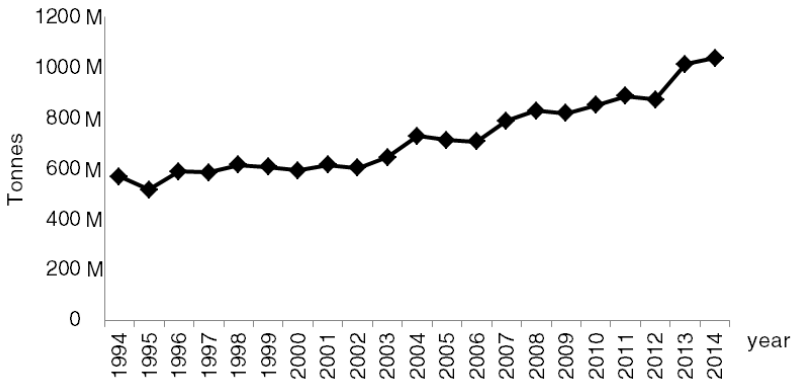

Figure 9. Maize production in the world, 1994-2014 (FAOSTAT, 2015).

However, maize is still a staple food for one third of the world's population, namely in sub-Saharan Africa, Southeast Asia, and Latin America, providing an estimated 15% of the world protein and 20% of the world calories. It is especially important in Africa, where its consumption ranges from 52 to 328 g/person/day. Most of these countries suffer from micronutrient

deficiencies, which makes maize a target crop for nutritional programs due to its low production costs and the high consumption of maize-derived food products (Nuss and Tanumihardjo, 2010; Ranum *et al.*, 2014). The nutritional value of maize grain can be enhanced by fortification (i.e. through the addition of multivitamin premixes to maize flour) or biofortification (i.e. through conventional breeding or genetic engineering). Nevertheless, fortification of maize flours is not usually possible in developing countries as it implies food processing, packaging and a distribution network, making the product economically inaccessible to poor people that consume most of their daily energy requirements from maize (Gómez-Galera *et al.*, 2010).

3.1. High-carotenoid maize

High-carotenoid (HC) maize has been developed to increase PVA in the diet and thus address VAD in at-risk populations in a sustainable manner. The South African elite white maize inbred M37W, which lacks carotenoids in the endosperm because of the absence of the enzyme phytoene synthase 1 (PSY1), was used as the basis to create the HC maize. HC maize was generated through the introduction of two transgenes under the control of endosperm-specific promoters: *Zea mays* phytoene synthase 1 (*Zmpsy1*), under the control of the wheat LMW glutenin promoter, and *Pantoea ananatis crtI* encoding carotene desaturase (*PacrtI*), under the control of the barley D-hordein promoter. This recreated the entire pathway from GGPP to lycopene in the maize endosperm (Fig. 4). Consequently, HC maize accumulates 88 μg/g dry weight (dw) of total carotenoids, with high levels of β-carotene, β-cryptoxanthin, lutein and zeaxanthin (Fig. 10) (Zanga *et al.*, 2016a; Zhu *et al.*, 2008). The endosperm color difference between M37W and HC, which reflects the enhanced carotenoid content in the latter, can be seen in Figure 11.

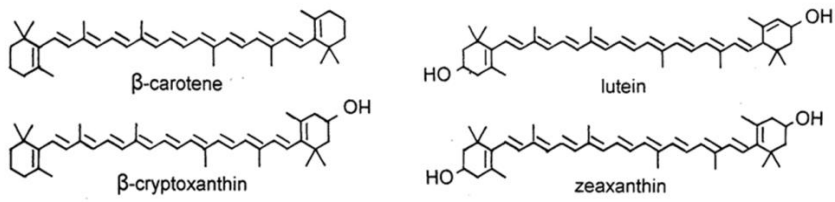

Figure 10. Main carotenoids present in high-carotenoid (HC) maize.

Figure 11. White maize inbred M37W (above) and transgenic high-carotenoid (HC) (below) cobs, the latter showing significant increases in the levels of carotenoids in the transgenic HC cob.

Multivamin maize is an improved version of HC maize, registered in Spain as the protected variety Carolight® (Zanga *et al.*, 2016b). Three different vitamin metabolic pathways were engineered simultaneously to transform M37W maize with four transgenes: *Zmpsy1* and *PacrtI,* representing the carotenoid pathway; *Osdhar* (rice dehydroascorbate reductase), representing the ascorbate pathway; and *EcfolE* (bacterial GTP cyclohydrolase 1), representing the folate pathway. The endosperm thus accumulates 169–fold more β-carotene, as well as other nutritionally important carotenoids (lycopene, lutein and zeaxanthin); 6–fold more ascorbate (vitamin C); and double the normal amount of folate (vitamin B9), in each case compared with the near isogenic M37W maize (Naqvi *et al.*, 2009). A further improved version has been created to be resistant to lepidopteran insect pests due to the presence of one or more insecticidal Bt transgenes (*cry1Ac*, *cry1Ca* and *vip3Aa*) (Zanga *et al.*, 2016b).

3.2. Mycotoxins in maize

Several pathogenic and opportunistic fungal species can colonize and induce disease in maize and, consequently, they can also produce toxic secondary metabolites in infected tissue, so-called mycotoxins (Logrieco *et al.*, 2007). **Mycotoxins** are low-molecular weight natural products produced as secondary metabolites by filamentous fungi, which constitute toxigenically and chemically heterogeneous compounds that are grouped together only

because they can cause disease and death in human beings and other vertebrates (Bennett, 1987). This toxic effect on human and animal health is known as mycotoxicosis, but toxin-producing fungus is not necessarily involved in the diseases that it causes (Marín *et al.*, 2013). Recently, it has been pointed out that up to 80% of global agricultural commodities could be contaminated with mycotoxins, although the majority of the samples analyzed were within the maximum levels or guidance levels established by the EU (Kovalsky *et al.*, 2016; Streit *et al.*, 2013).

Fungal toxins of interest are mainly produced by fungal species within the genera *Fusarium*, *Aspergillus*, *Penicillium, Alternaria* and *Claviceps,* which can grow on a variety of crops, including cereals, and can occur at any time along the food chain, including pre-harvest, harvest, drying, storage and manufactured products. Consequently, their toxins could potentially appear in different foods and feeds. The main mycotoxins found in food are aflatoxins (AFs), produced by *Aspergillus* spp.; ochratoxin A (OTA) produced by both *Aspergillu*s and *Penicillium*; trichothecenes type A (e.g. HT-2 and T-2 toxin) and trichothecenes type B (e.g. deoxynivalenol – DON –, nivalenol – NIV –), zearalenone (ZEA), fumonisins B_1 (FB_1) and B_2 (FB_2), and some recently-discovered *Fusarium* metabolites collectively known as emergent mycotoxins, such as moniliformin (MON), beauvericin, enniatins and fusaproliferin, produced mainly by *Fusarium* spp.; ergot alkaloids produced by *Claviceps*; and alternariol and other toxins produced by *Alternaria* spp. (Marín *et al.*, 2013).

Maize is the most important source of fumonisin intake in human and animal diets (Table 2), as well as its resulting milling fractions, such as grits and flour used for further processing (Marín *et al.*, 2013). Fumonisin contamination in maize is directly associated with *Fusarium* pink ear rot or pink fusariosis, mainly produced by *F. verticilliodes* (teleomorph *Gibberella moniliformis*), *F. proliferatum* (teleomorph *G. intermedia*) and *F. subglutinans* (teleomorph *G. subglutinans*). Another common disease in maize is *Fusarium* red ear rot or red fusariosis, mainly produced by *F. graminearum* (teleomorph *G. zeae*), which produces several mycotoxins, mainly trichothecenes and zearalenones. The fungal prevalence depends primarily on environmental conditions; *Fusarium* pink ear rot is related to drier and warmer climates, whereas *Fusarium* red ear rot is associated with rainfall and low temperatures during summer and early fall (Logrieco *et al.*, 2007).

Table 2. Fumonisin B_1 (μg/kg) in food samples in the European Union (Marín *et al.*, 2013; SCOOP, 2003). [a] Samples < LOD (limit of detection) were given the LOD/2 value.

Sample	N samples	N samples > LOD	LOD	Mean[a]	Maximum
Maize grain	801	534 (67%)	5–50	346.4	10200
Maize grits	172	98 (57%)	5–30	347.6	4800
Maize flour	110	87 (97%)	5–50	408.5	4766
Maize flakes	274	125 (46%)	0.2–500	31.5	1092
Sweet maize	145	13 (9%)	5–500	12.4	81
Polenta	29	29 (100%)	20–50	182.2	752

Fungal infection mainly depends on climatic conditions, maize genotypes, agricultural practices, spoilage and other fungal diseases (Ariño *et al.*, 2009; Soriano and Dragacci, 2004). The main factors that affect the life cycle of mycotoxigenic molds (i.e. germination, growth, sporulation and mycotoxin production) are water availability and temperature (Magan *et al.*, 2011). Temperatures from 15–20 °C and high kernel moisture until 80 days after silking favor natural grain infection by *Fusarium verticillioides* rather than by *Aspergillus* species (Cao *et al.*, 2013). Moderate rainfall at maize silking combined with warm temperatures seems to be necessary for *Fusarium* infection, which can also be promoted by insect damage. *F. verticillioides*, which appears to be endemic in maize grains, colonizes maize grains before other fungi (Marín *et al.*, 2004). On the other hand, grain infection by *Aspergillus flavus* is usually delayed until maturity, and it is not usually observed before early dent stage (Giorni *et al.*, 2016). At high temperature and low water activity, *A. flavus* is likely to be the dominant fungal species in the field and during post-harvest (Giorni *et al.*, 2016). Fungal growth and mycotoxin production are closely linked to environmental conditions, which is why climate change could result in higher pre-harvest levels of mycotoxins (Battilani *et al.*, 2016). Maize grains will probably be more predisposed to fungal infection and mycotoxin contamination under warmer temperatures combined with greater rainfall or drought, which increase plant stress (Wu *et al.*, 2011).

Maize varieties and agricultural practices are important factors in the prevention and control of mycotoxins. Maize genotypes can be more or less susceptible to mycotoxin contamination, but this susceptibility is also influenced by the cultivar area, the climatic conditions and the crop

management (Ariño *et al.*, 2009; Soriano and Dragacci, 2004). For instance, Bt maize varieties can indirectly decrease fungal infection, and the resulting fumonisin contamination, by reducing insect pests (for further information see Annex III). Apart from that, to date there are no maize genotypes highly resistant to fumonisin contamination, which makes the application of good agricultural practices necessary to prevent mycotoxin contamination (Ariño *et al.*, 2009). Dry planting, minimum tillage, hydric stress, high plant density, high nitrogen fertilization and delayed sowing and harvest dates are not recommended practices as they have been related to higher fumonisin levels in maize. In contrast, wet planting, early sowing date, pest control, hybrid maturity, and removal of residue from the previous crop can significantly contribute to fumonisin reduction (Ariño *et al.*, 2009; Blandino *et al.*, 2009). This highlights the importance of an integrated field program to prevent mycotoxin contamination.

Fumonisins (FBs) were named after the isolation in 1988 of FB_1 from *F. moniliforme* (now renamed *F. verticillioides*) by South African researchers (Bezuidenhout *et al.*, 1988; Seifert *et al.*, 2003). FB_1 is the most common as well as the most toxigenic fumonisin found in maize, but fumonisins B_2 and B_3 (FB_2 and FB_3) are common co-contaminants (Desjardins, 2006). Since their discovery in 1988, in connection with high human esophageal cancer rates in the former Transkei region of South Africa, many reports have described their deleterious effects. Consumption of fumonisin-contaminated maize has a number of toxic effects on domestic animals, such as leukoencephalomalacia in equines or pulmonary edema, reduced weight gain, and liver damage in pigs. A correlation with cancer has been found in humans, especially in areas where maize is the staple diet (Marasas, 2001; Wu *et al.*, 2014). The mode of action of FBs is directly or indirectly linked to sphingolipid metabolism, as fumonisin inhibits the enzyme ceramide synthase, which is critical for biosynthesis of sphingolipids that have multiple functions in the body (Marasas *et al.*, 2004; Wu *et al.*, 2014). Taking into account the differences in dietary habits and food safety standards existing between fully developed and developing countries, human exposure to FBs may be higher in the latter, as in many parts of Africa, South and Central America, and Asia, the population consumes a high percentage of maize (JECFA, 2001).

Other mycotoxins may be present in maize alone or together with FBs, including AFs, DON, ZEA, and some emergent mycotoxins (Marín *et al.*, 2013). Among *Fusarium*-produced toxins, co-occurrence is frequently

observed for compounds such as FBs, DON and ZEA, and synergistic effects of *Fusarium* have been reported (Kovalsky *et al.*, 2016). Figure 12 shows the molecular structures of the main mycotoxins found in maize.

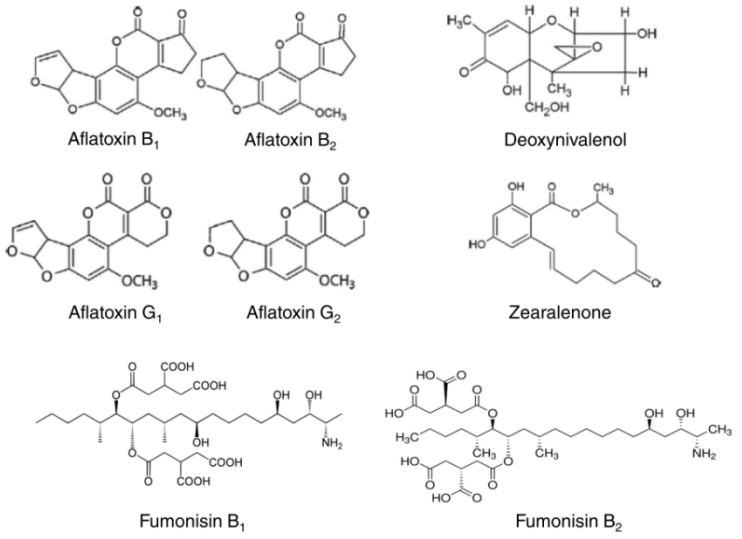

Figure 12. Molecular structures of major mycotoxins found in maize.

AFs are hepatotoxic, carcinogenic, immunosuppressive and antinutritional contaminants, mainly produced by *Aspergillus flavus*, which synthesizes type B AFs as well as cyclopiazonic acid (CPA), depending on the strain, and by *A. parasiticus*, which synthesizes both type B and type G AFs, but not CPA (IARC, 2012; Marín *et al.*, 2013). The ingestion of aflatoxin-contaminated products can result in two forms of aflatoxicosis, acute and chronic. Acute severe intoxication is produced when the food is contaminated with high levels of AFs, resulting in direct liver damage and subsequent illness or death. Aflatoxin-contaminated maize was related to a large number of acute human aflatoxicosis in developing countries. As highly contaminated feed is more frequent, acute aflatoxicosis is more common in animals, although the susceptibility varies among species (Marín *et al.*, 2013; Williams *et al.*, 2004; Wu *et al.*, 2014). Chronic subsymptomatic exposure occurs when relatively small amounts of AFs are consumed over an extended period. In farm and laboratory animals, chronic exposure to AFs compromises immunity, which results in suppression of the cell-mediated immune responses. The nutritional status can also be compromised, with a reduction in the growth or other productivity parameters (Marín *et al.*, 2013; Williams *et al.*, 2004).

DON and ZEA are mainly produced by *F. graminearum* and *F. culmorum* and are often found as co-contaminants in maize. DON is a member of the trichothecenes family, but is not as toxic as other trichothecenes such as T-2 or HT-2 toxins. Colloquially known as vomitoxin, it presents toxic effects in animals that range from feed refusal, vomiting and nausea, to immunosuppression and loss of productivity, whereas in humans it can cause acute effects related to gastrointestinal illness (Marin *et al.*, 2013; Sobrova *et al.*, 2010). On the other hand, ZEA and some of its related metabolites competitively bind to oestrogen receptors. Consequently, its toxicity is related to reproductive problems in specific animal species (e.g. pigs and laboratory animals) and possibly in humans. In animals, chronic exposure to ZEA is associated with carcinogenicity, genotoxicity, reproductive toxicity, endocrine effects, and immunotoxicity, pig being one of the most susceptible animals (EFSA, 2011; Marín *et al.*, 2013). Among the so-called emergent mycotoxins, the most relevant is MON, a very strong acid produced by several *Fusarium* species (*F. avenaceum*, *F. tricinctum*, *F. proliferatum*, *F. subglutinans*, and *F. verticillioides*), which is an inhibitor of thiamine pyrophosphate-dependent enzymes, pyruvate dehydrogenase being the most studied (Marín *et al.*, 2013; Peltonen *et al.*, 2010).

According to the International Agency for Research on Cancer (IARC) (Table 3), from among the mycotoxins found to occur in maize only AFs (AFB_1, AFB_2, AFG_1 and AFG_2) and FB_1 are classified as carcinogenic (group 1) and possibly carcinogenic to humans (group 2B), respectively, while the rest are not classifiable as to carcinogenicity in humans (group 3) (IARC, 1993, 2002, 2012). AFB_1 is considered the most toxic aflatoxin, and it is also the most carcinogenic natural compound (IARC, 2012). As stated by the annual report of the Rapid Alert System for Food and Feed (RASFF, 2016), in 2015 mycotoxins were the main hazard in border rejection notifications in the EU, AFs being the primarily responsible mycotoxin. Among RASFF notifications, there were five notifications due to the presence of FBs (of which one combined with a high level of AFs) in maize and maize products intended for human consumption and another five notifications owing to the presence of AFs in maize and maize products intended for feed. Natural co-occurrence for AFs and FBs in maize has been reported in various regions of the world, observing the highest co-occurrence rate in stored maize (Hove *et al.*, 2016). Therefore, given the toxicological significance of AFB_1 and FB_1, and the toxicological effects caused by combined exposure, it is important to analyze both mycotoxins in maize-derived products.

Table 3. IARC classification of the main mycotoxins. Abbreviations: AFs, aflatoxins, AFM_1, aflatoxin M_1; FBs, fumonisins; OTA, ochratoxin A; DON, deoxynivalenol, NIV, nivalenol, PAT, patulin, ZEA, zearalenone (IARC, 1993, 2002, 2012).

IARC number	Definition	Mycotoxins
1	The mycotoxin is carcinogenic to humans	AFs
2A	The mycotoxin is probably carcinogenic to humans	_
2B	The mycotoxin is possibly carcinogenic to humans	AFM_1, FBs, OTA, sterigmatocystin
3	The mycotoxin is not classifiable as to its carcinogenicity to humans	DON, NIV, PAT, T-2/HT-2, ZEA, citrinin, fusarenon-X
4	The mycotoxin is probably not carcinogenic to humans	_

4. Poultry production

Chickens (*Gallus gallus* subsp. *domesticus*) were probably domesticated 7,500 years ago in Northeast China from where they spread to Europe and Africa, and were then brought to America by European settlers. In the late 1920s, poultry started to be considered as a source of meat rather than a by-product of egg production, owing to breeding and genetic techniques. The term **poultry** applies to domesticated birds that are normally slaughtered and prepared for market, including chickens, turkeys, ducks and quails, among others. While the term **broiler** refers to a young chicken suitable for cooking, its origin is related to a cooking technique based on *broiling* the chicken over an open fire (Leeson and Summers, 2000; Scanes *et al.*, 1992).

It is estimated that world population will grow from 7 billion in 2015 to 10 billion in 2050 (UN, 2015). So far, the poultry industry has been able to meet increased demands for human food thanks to improved production efficiency, which allows chicken to be an economical source of animal protein, and the adaptability for further processing (e.g. chicken nuggets, chicken wings and mechanically deboned meat), which has also contributed to improving the production efficiency (Leeson and Summers, 1997). Poultry meat meets consumer demands due to its nutritional composition (e.g. high-quality protein and low fat) and its good eating properties, without forgetting its economical price (Scanes *et al.*, 1992). Figure 13 shows the retail cuts of chicken.

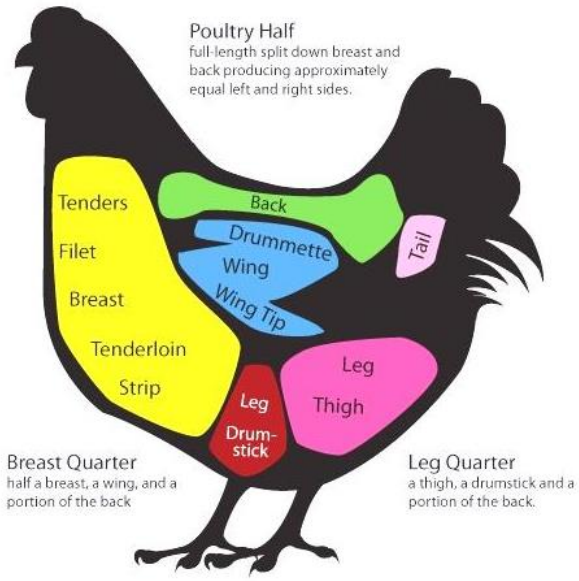

Figure 13. Chicken retail cuts (BA-farms, 2017).

The chicken industry has experienced a continuous increase in the last 20 years, reaching a production of 21 billion chickens in 2014 (Fig. 14). As a result, it has increased the demand for maize for livestock feed to support increased meat production. Maize is the most commonly used cereal grain in the intensive poultry industry, as it contributes approximately 65% of the apparent metabolizable energy and 20% of the protein in a poultry diet. In contrast to other grain types, maize is rich in starch and has a relatively low concentration of water-soluble non-starch polysaccharides and other antinutritional factors (e.g. phytin, tripsin inhibitors and lectins). For this reason, maize grains have a high digestibility and can be included as up to 65% of the chicken diet (Cowieson, 2005; Leeson and Summers, 2000).

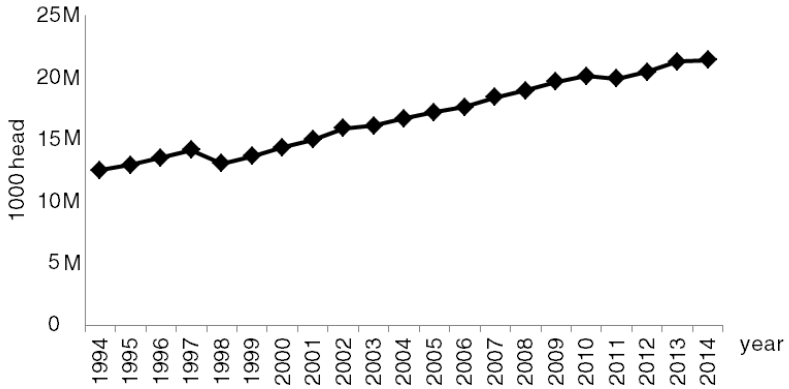

Figure 14. Chicken production in the world, 1994-2014 (FAOSTAT, 2016).

The majority of chickens used for commercial meat production in the Western world are homozygous for a recessive allele, the yellow skin allele, whereas white skinned chickens carry the dominant allele. Thus, yellow skin is caused by tissue-specific regulatory mutations that inhibit expression of the β-carotene-9',10'-dioxygenase 2 (BCDO2) enzyme in skin, but not in other tissues. As BCDO2 cleaves colorful carotenoids into colorless apocarotenoids, a reduction in expression of this gene produces yellow skin (Eriksson *et al.*, 2008). In chickens, as in mammals, PVA carotenoids are converted into retinol by BCDO1, mainly in the intestinal mucosa (Álvarez *et al.*, 2015). Recently, it has been shown that xanthophyll supplementation could modulate carotenoid and retinoid metabolism in hens and chicks, as lutein and zeaxanthin are involved in gene expression associated with carotenoid cleavage enzymes and retinoid metabolism (Gao *et al.*, 2016).

Therefore, the expression of yellow skin is influenced by the amount of carotenoids in the feed. Skin color plays a major role in consumer preference for poultry meat as it is the first quality attribute that the consumer can evaluate. Many consumers in North America and the Asia Pacific markets prefer a golden skin color because it is associated with better health. Thus, natural or synthetic pigments have to be added to enhance the yellow color (Tarique *et al.*, 2013; Williams, 1992). The United States, Brazil, China, Russia and Mexico are the major producers of chicken meat in the world (Table 4).

Table 4. Chicken meat production in 2014, by country (FAOSTAT, 2015).

Country	Chicken meat production in 2014 (MT/year)
United States of America	18
Brazil	13
China	12
Russia	4
Mexico	3
India	3
Japan	2
Iran	2
Indonesia	2
Argentina	2

Golden skin pigmentation for poultry meat is preferred by the majority of these markets. Thus, more carotenoids for use as feed colorant will be required, which can increase the price of the final product. Commercially, carotenoids are used as colorants (in food and feed) and in nutritional supplements, with an estimated global market of $US 1.5 billion in 2014, which is expected to reach nearly $US 1.8 billion in 2019 (BCC Research, 2015). Carotenoids commercially used for poultry feed supplementation can be obtained by chemical extraction from natural sources (e.g. *Tagetes erecta* and *Capsicum annuum*), by industrial fermentation (e.g. *Blakeslea trispora)* or by chemical synthesis (e.g. β-carotene) (Berman *et al.*, 2015). The major carotenoids used in chicken nutrition include yellow xanthophylls (e.g. lutein and zeaxanthin) and red xanthophylls (e.g. capsanthin and canthaxanthin), which are usually combined according to the color desired by the market (Breithaupt, 2007). These are approved feed additives for poultry diets in the EU (Table 5).

Table 5. List of color additives authorized by the European Union for use in poultry and laying hen diets.

Additive (EC No)	Animal species	Maximum content (mg/kg) of complete feed (12 % moisture)	Legislation
Capsanthin (E 160c)	Poultry with the exception of turkeys	80[a]	EC, 1985
β-apo-8'-carotenal (E 160e)	Poultry	80[a]	EC, 1985
β-apo-8' carotenoic acid ethyl ester (E 160f)	Poultry	80[a]	EC, 1985
Lutein (E 161b)	Poultry	80[a]	EC, 1985
Cryptoxanthin (E 161 c)	Poultry	80[a]	EC, 1985
Zeaxanthin (E 161h)	Poultry	80[a]	EC, 1985
Citranaxanthin (E 161i)	Laying hens	80[a]	EC, 1985
Canthaxanthin (E 161g)	Poultry Laying hens	25[b] 8[b]	EC, 2015

[a] Alone or with other carotenes and xanthophylls.

[b] The mixture of canthaxanthin with other carotenoids and xanthophylls shall not exceed 80 mg/kg of complete feed.

It is interesting to note that the use of astaxanthin as feed additive is only authorized for salmon and trout (European Commission, 2008). According to the European legislation concerning additives for use in animal nutrition, carotenoids, depending on their function, could be considered as **sensory additives**, defined as any substance, the addition of which to feed improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals, or as **nutritional additives**, which include vitamins, pro-vitamins and chemically well-defined substances having a similar effect (European Commission, 2003).

4.1. Carotenoid metabolism in chickens

After ingestion of feed, carotenoids are released from the matrix by digestive enzymes and then carotenoids, as well as other feed lipids, are emulsified in the presence of bile salts to form oil droplets. As a result of lipase action, carotenoids are transferred from oil droplets to mixed micelles (Surai *et al.*, 2001). Carotenoid can be absorbed by passive diffusion across the brush border membranes of the intestinal mucosal epithelium (Fig. 15), which includes the migration of mixed-micelles to the membranes of intestinal mucosal cells, incorporation into lipoprotein particles and release into the portal vein system by portomicrons (i.e. the lipid rich lipoproteins released from the intestinal cells), and then carotenoids can be directly delivered to the liver and other tissues (Surai *et al.*, 2001). In recent years, it has been shown that carotenoid uptake is partly mediated by facilitated diffusion, as carotenoids can be actively absorbed by membrane transporters involved in the lipid metabolism (Fig. 15), such as SCARB1 and cluster determinant 36 (CD36) (Jlali *et al.*, 2014; Kotake-Nara and Nagao, 2011).

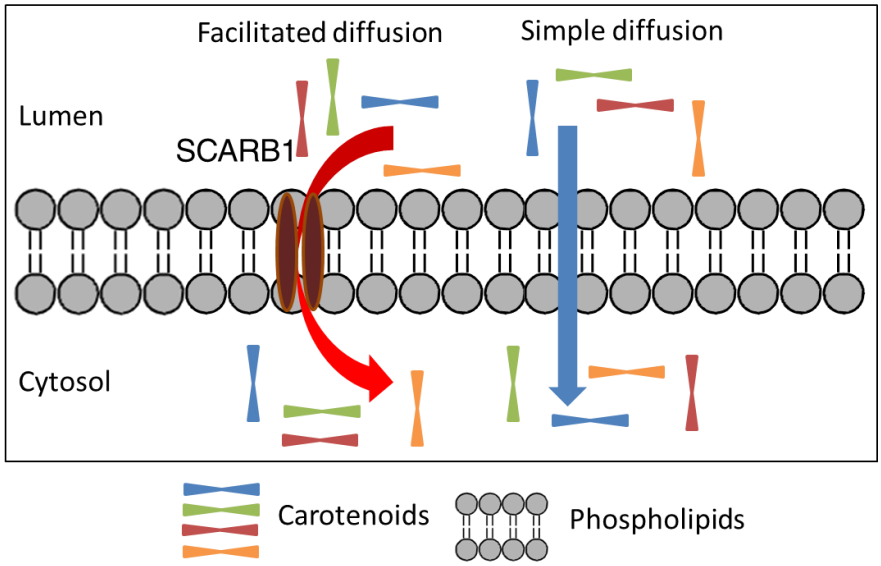

Figure 15. Intestinal absorption of the dietary carotenoids in the intestinal lumen of chickens. Adapted from Kotake-Nara and Nagao (2011).

Among the proteins involved in carotenoid metabolism, BCDO1 is mainly responsible for the symmetrical cleavage of β-carotene into 2 retinal molecules (and then retinal can further undergo reversible reduction to

retinol or oxidation to retinoic acid), whereas BCDO2 is mainly responsible for the asymmetric cleavage of carotenoids into apocarotenoids (Jlali *et al.*, 2014; Surai *et al.*, 2001). The most important factor delimiting the efficiency of carotenoid assimilation from the feed is carotenoid absorption in the intestine. However, if carotenoids are presented as esterified forms instead of free alcohol form, the carotenoid ester hydrolysis by pancreatic esterases is also important (Surai *et al.*, 2001).

Vitamin A plays an important role in both adult and embryo chicken development and can be efficiently stored in the liver and yolk of chickens thanks to its lipophilic nature (Yuan *et al.*, 2014). Vitamin A and carotenoid metabolism in chickens is closely related to the equivalent processes in humans, and therefore chickens are also susceptible to VAD with similar symptoms. Dietary VAD decreased performance, caused infertility or impaired reproduction, and depressed the immune system in poultry (NRC, 1994; Pretorius and Schönfeldt, 2013; Yuan *et al.*, 2014). Thus, vitamin A supplementation could ensure immunity, growth and development. For this reason, poultry feed formulations usually contain vitamin A at different levels according to age (Yuan *et al.*, 2014). In general, for maize-based feed, broiler breeding companies recommend vitamin A supplementation up to 12,000 IU/kg in starter diets and up to 10,000 IU/kg in grower diets (Aviagen, 2014). Synthetic retinyl esters are usually the main source of vitamin A in the poultry industry, whereas carotenoids only provide a small part of total vitamin A requirement (Surai *et al.*, 2001).

Taking into account these requirements from the poultry industry, novel sources of carotenoids in the poultry diet could contribute to enhancing both animal appearance and health, without adding color additives and reducing the quantity of vitamin A fortificants.

5. Main regulatory issues in the European Union

5.1. Genetically modified food and feed

- Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorization of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. Official Journal of the European Union L157: 1–48.

- Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Official Journal of the European Union L268: 24–28.
- Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Union L268: 1–23.
- Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L106: 1–38.
    - Directive (EU) 2015/412 of the European Parliament and of the Council of 11 March 2015 amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory. Official Journal of the European Union L68: 1–8.
    - Directive 2008/27/EC of the European Parliament and of the Council of 11 March 2008 amending Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, as regards the implementing powers conferred on the Commission. Official Journal of the European Union L81: 45–47.

5.2. Animals used for scientific purposes

- Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276: 33–79.
- Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). Official Journal of the European Union L197: 1–89.

Other regional and national laws which it is necessary to fulfill:

- Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en

experimentación y otros fines científicos, incluyendo la docencia. Boletín Oficial del Estado 34: 11370–11421 (Spain).

- Ley 32/2007, de 7 de noviembre, para el cuidado de los animales, en su explotación, transporte, experimentación y sacrificio. Boletín Oficial del Estado 268: 45914-45920 (Spain).
  - Ley 6/2013, de 11 de junio, de modificación de la Ley 32/2007, de 7 de noviembre, para el cuidado de los animales, en su explotación, transporte, experimentación y sacrificio. Boletín Oficial del Estado 140: 44289–44292 (Spain).
- Decreto 214/1997, de 30 de julio, por el que se regula la utilización de animales para experimentación y para otras finalidades científicas. Diari Oficial de la Generalitat de Catalunya 2450: 9169–9175 (Catalonia).
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OBJECTIVES AND WORK PLAN

High-carotenoid (HC) maize was generated through genetic engineering to specifically increase the levels of carotenoids present in the maize endosperm, using as the basis a South African white maize, which is practically devoid of carotenoids. Consequently, the intake of provitamin A (PVA) carotenoids in the diet would be increased and could thus address vitamin A deficiency in at-risk populations in developing countries. The effects that its use as raw material could have on feed and food are unknown. Therefore, the main objective of this Doctoral Thesis is to evaluate its possible utilization as feed and food ingredient. The general objectives of this Doctoral Thesis are described below. To achieve each goal, it was necessary to set specific objectives.

I. Evaluate, for three consecutive harvest seasons, the susceptibility to fungal infection and fumonisin and aflatoxin contamination of HC maize and its near isogenic line in open field experiments (**Chapter I**).

   i. Assess whether there are differences in fungal incidence and the fungal species involved between HC maize and its near isogenic line.

   ii. Investigate whether the carotenoid content could play a role in fumonisin and aflatoxin contamination.

II. Evaluate whether HC maize could be a suitable replacement for color additives in poultry feed (**Chapter II**):

   i. Compare the productivity and health parameters among chickens fed on different diets, based on HC maize, its near isogenic line and a commercial yellow maize (supplemented with natural color additives).

   ii. Investigate the color evolution among chickens fed on different diets, based on HC maize, its near isogenic line and a commercial yellow maize (supplemented with natural color additives), and the levels of carotenoids and their derivatives in feed, serum and liver.

   iii. A further aim was to obtain chicken meat to carry out the trial related to objective IV.

III. Assess the bioavailability of PVA carotenoids (β-carotene and β-cryptoxanthin) provided as intrinsic components of HC maize (**Chapter III**).

i. Compare the bioavailability of PVA carotenoids supplied by HC maize or by its near isogenic line supplemented with synthetic or natural color additives.

ii. Ascertain whether zeaxanthin, a major carotenoid in HC maize, may play a role in carotenoid absorption.

iii. Evaluate the productivity parameters, color evolution and distribution of carotenoids and their derivatives among chickens fed on diets based on HC maize and its near isogenic line supplemented with synthetic or natural color additives.

IV. Investigate the effect of HC maize on chicken meat quality and shelf life (**Chapter IV**).

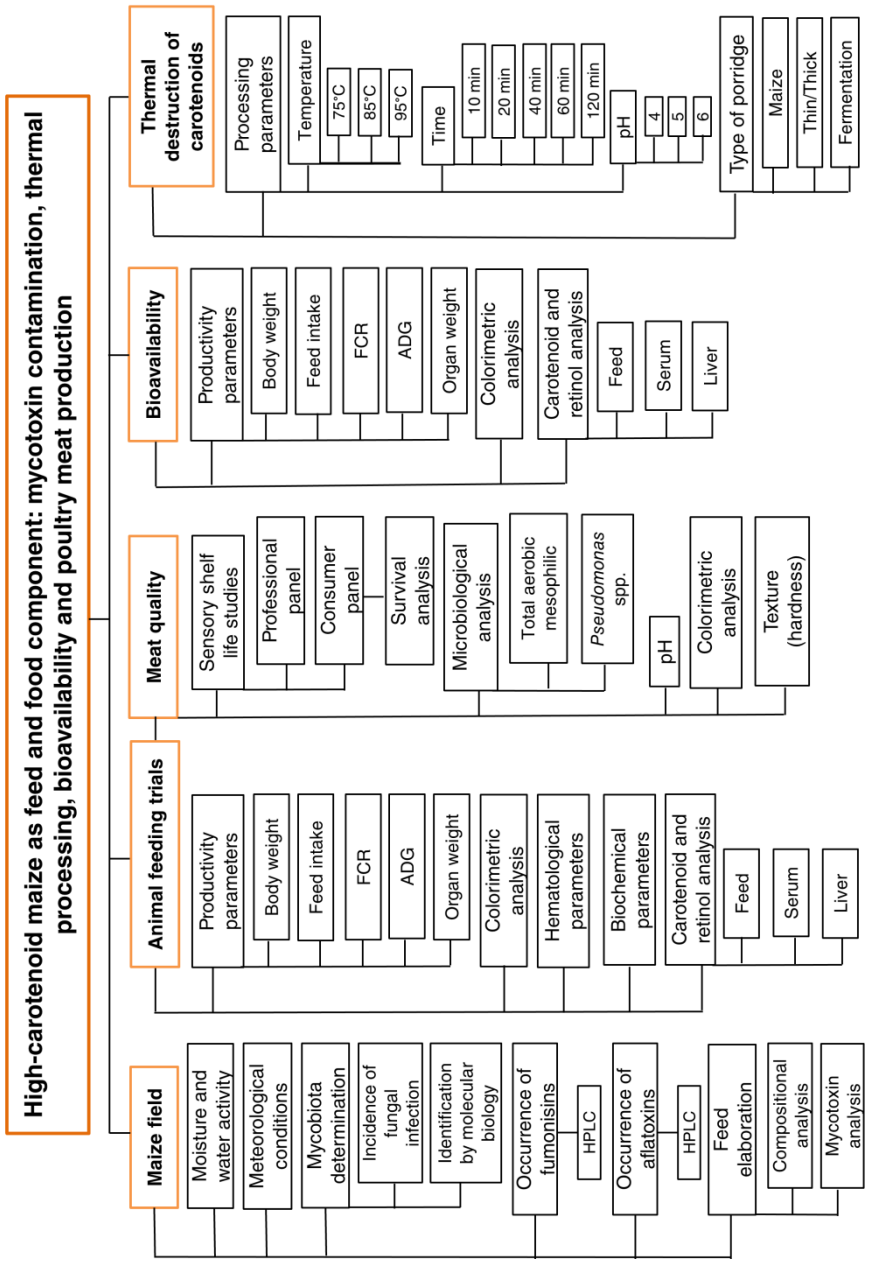
i. Ascertain the sensory shelf life of chicken meat obtained from chickens fed on different diets based on HC maize, its near isogenic line and a commercial yellow maize (supplemented with natural color additives).

ii. Evaluate the quality of chicken meat through physical (pH, color and texture) and microbiological analyses (*Pseudomonas* spp. and total aerobic mesophilic).

V. Ascertain the optimal conditions for preserving carotenoids in HC maize when it is cooked (**Chapter V**).

i. Investigate the effect of processing parameters (temperature, cooking time and pH) on total carotenoid content of HC maize.

ii. Assess the effect of different home-cooking techniques (thin or thick, unfermented or fermented porridges) on final carotenoid content in maize porridges obtained from HC maize and its near isogenic line.


High-carotenoid maize as feed and food component: mycotoxin contamination, thermal processing, bioavailability and poultry meat production
Maize field
Moisture and water activity
Meteorological conditions
Mycobiota determination
Incidence of fungal infection
Identification by molecular biology
Occurrence of fumonisins
HPLC
Occurrence of aflatoxins
HPLC
Feed elaboration
Compositional analysis
Mycotoxin analysis
Animal feeding trials
Productivity parameters
Body weight
Feed intake
FCR
ADG
Organ weight
Colorimetric analysis
Hematological parameters
Biochemical parameters
Carotenoid and retinol analysis
Feed
Serum
Liver
Meat quality
Sensory shelf life studies
Professional panel
Consumer panel
Survival analysis
Microbiological analysis
Total aerobic mesophilic
Pseudomonas spp.
pH
Colorimetric analysis
Texture (hardness)
Bioavailability
Productivity parameters
Body weight
Feed intake
FCR
ADG
Organ weight
Colorimetric analysis
Carotenoid and retinol analysis
Feed
Serum
Liver
Thermal destruction of carotenoids
Processing parameters
Temperature
75°C
85°C
95°C
Time
10 min
20 min
40 min
60 min
120 min
pH
4
5
6
Type of porridge
Maize
Thin/Thick
Fermentation

RESULTS AND DISCUSSION

CHAPTER I

The effect of enhanced carotenoid content of transgenic maize grain on fungal colonization and mycotoxin content

Joana Díaz-Gómez, Sonia Marín, Carmina Nogareda, Vicente Sanchis and Antonio J. Ramos

Mycotoxin Research (2016), 32 (4): 221–228

The effect of enhanced carotenoid content of transgenic maize grain on fungal colonization and mycotoxin content

Joana Díaz-Gómez [a], Sonia Marín [a], Carmina Nogareda [b], Vicente Sanchis [a] and Antonio J. Ramos [a]

[a] Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, Av. Rovira Roure 191, Lleida, 25198, Spain;
[b] Animal Science Department, University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, Lleida, 25198, Spain

Abstract

Novel strategies that address vitamin A deficiency have been developed, such as high-carotenoid maize, a biofortified transgenic maize line rich in carotenoids generated by genetic transformation. The South African white maize inbred (M37W), which is devoid of carotenoids, was engineered to accumulate high levels of β-carotene (provitamin A), lutein and zeaxanthin. Maize seeds contaminated with fumonisins and other mycotoxins pose a serious threat to both humans and livestock. During three consecutive harvests, the fungal incidence and the fumonisin and aflatoxin content of maize seeds grown in an experimental field in Catalonia (Northeastern Spain) were evaluated. Fungal infection was similar in high-carotenoid maize and its near isogenic line, with *Fusarium verticillioides* being the most prevalent fungus in all the harvests. Neither *Aspergillus* spp. nor aflatoxin contamination was found. Fumonisin levels were lower in high-carotenoid than in its near isogenic line, but this reduction was statistically significant in only 2 of the 3 years of study. Our results suggest that high carotenoid content reduces fumonisin levels in maize grains.

Keywords

Maize, carotenoids, fumonisins, aflatoxins, *Fusarium*

1. Introduction

In many developing countries, populations subsist on a cereal-dominated diet with low levels of nutrients, including β-carotene. Chronically

insufficient vitamin A intake in the diet can lead to disorders such as xerophthalmia, anemia and weakened resistance to infection. It has been estimated that vitamin A deficiency (VAD) affects up to one third of the world's pre-school-age children and up to 15% of pregnant women. VAD is prevalent worldwide but is particularly severe in Africa and South-East Asia (WHO, 2009).

In sub-Saharan Africa, white maize is the predominant food maize used, while yellow maize, with a higher carotenoid content (but with no provitamin A – PVA – carotenoids), is mainly used as animal feed (Naqvi *et al.*, 2009). The South African white endosperm maize inbred M37W, which lacks carotenoids in the endosperm because of the absence of the enzyme phytoene synthase (necessary for the biosynthesis of these metabolites), was used as a basis to create the high-carotenoid (HC) maize through combinatorial nuclear transformation (as described in Zhu *et al.*, 2008). *Zmpsy1* (*Zea mays* phytoene synthase 1) and *PacrtI* (*Pantoea ananatis* phytoene desaturase) controlled by endosperm-specific promoters were introduced into M37W inbred to generate the HC maize. The maize line used in the current experiments is able to provide the recommended daily intake of PVA in 200 g of grain (Naqvi *et al.*, 2009).

Mycotoxins occurring in food commodities are low-molecular-weight natural products synthesized as secondary metabolites by filamentous fungi. The most important toxins are produced by species in the genera *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*, which can grow on a variety of crops, including cereals, and can enter the food chain during preharvest, harvest, drying, or storage, and even in manufactured products, thus offering many opportunities to contaminate food and feed (Shephard, 2008). Maize (*Zea mays* L.) seeds are often contaminated with fumonisins (FBs), produced primarily by *Fusarium verticillioides* and *F. proliferatum*. However, other mycotoxins may frequently be present alone or in combination with FBs, including aflatoxins (AFs), deoxynivalenol, zearalenone, and some recently discovered *Fusarium* metabolites collectively known as emergent mycotoxins, such as moniliformin, beauvericin, enniatins and fusaproliferin. AFs are mainly produced by *Aspergillus flavus*, which synthetizes type B aflatoxins as well as cyclopiazonic acid, depending on the strain, and by *A. parasiticus*, which synthetizes both type B and type G aflatoxins but not cyclopiazonic acid (Desjardins, 2006; Marín *et al.*, 2013).

Fumonisin-contaminated maize is toxic, causing leukoencephalomalacia in equines (Kellerman *et al.*, 1990), pulmonary edema in swine (Harrison *et al.*, 1990) and cancer in humans, especially in areas where maize is the staple diet (Marasas, 2001). The mode of action of FBs is directly or indirectly linked to the effects of FBs on the sphingolipid metabolism (Wang *et al.*, 1991). Fumonisin B_1 (FB_1) is potentially carcinogenic in humans (group 2B), according to the International Agency for Research on Cancer (IARC, 2002). It is also the most prevalent *Fusarium* toxin in maize. Human exposure to FBs in maize is common worldwide. In 2001, several countries submitted information on the concentration of FBs in maize and maize-derived foods, and FBs were detected in more than 60% of all food products (JECFA, 2001).

AFs are hepatotoxic, carcinogenic, immunosuppressive and anti-nutritional contaminants found in some staple foods. Chronic exposure to AFs can compromise the immunity and nutritional status of farm and laboratory animals, reducing growth and productivity. AFs are potent carcinogens in all animal species studied thus far (mice, rats, hamsters, fish, piglets and chickens) (Williams *et al.*, 2004; Marin *et al.*, 2013). According to the International Agency for Research on Cancer (IARC, 2012), there is sufficient evidence to classify the main AFs (AFB_1, AFB_2, AFG_1 and AFG_2) as carcinogenic in humans (group 1). Aflatoxin B_1 (AFB_1) is considered the most potent aflatoxin and is also the most carcinogenic natural compound known.

Maize is the most important source of fumonisin contamination in foods, and infected kernels pose a serious threat to both humans and livestock, leading to the importance of evaluating the susceptibility to contamination of new maize varieties. It is unknown the vulnerability to fungal infection and mycotoxin contamination of HC maize. Thus, this work aims to evaluate, for three consecutive harvest seasons, the fungal incidence and the fumonisin and aflatoxin presence in HC maize and its near isogenic control line in open field experiments.

2. Materials and methods

2.1. Maize

The South African white maize inbred M37W (control maize), which is essentially devoid of carotenoids, and its engineered derivative (HC maize),

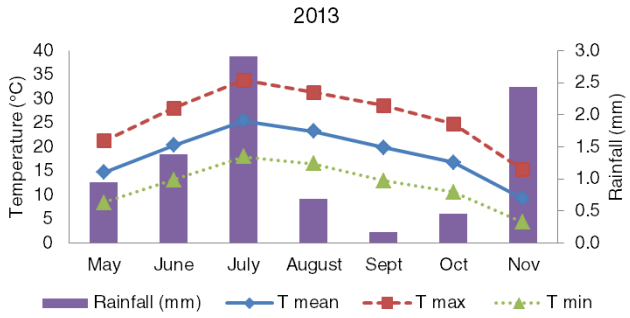
which accumulates high levels of β-carotene, lutein and zeaxanthin (Zhu *et al.*, 2008), were cultivated in an experimental field trial in Lleida (Catalonia, Northeastern Spain) during three consecutive years (2013, 2014 and 2015). Maize seeds were sown in May (2013 and 2014) or June (2015) and maize plants harvested in November in all the years of the study. The experimental field had a Latin square design with four replicates accommodating a two-way factorial. There were six-row plots and 28 plants per row, being the surface area of each plot 7 × 5 m^2. Every plot was randomly sampled at harvest to obtain a representative sample, and the samples from each plot were combined to obtain an aggregate sample of each type of maize for the mycobiota analysis. After harvest, maize cobs were dried at low temperature (35 °C) for 24 hours in a drying chamber, sampled according to the Commission Regulation No. 401/2006 (European Commission, 2006a), milled (Ras® Mill, Romer® Labs Inc., MO, USA) and frozen at -18 °C until analysis by high-performance liquid chromatography (HPLC).

2.2. Moisture and water activity determinations

The moisture content of the field samples was determined by oven drying according to the Association of Official Analytical Chemists method (AOAC International, 2005). The water activity (a_w) was determined using an Aqualab CX2T (Decagon Devices, Pullman, WA, USA).

2.3. Meteorological data

Meteorological data were obtained from the nearest meteorological station to the experimental field through the weather network of Catalonia (Servei Metereològic de Catalunya, 2016). Maximum, mean and minimum temperatures and rainfall from May to November were recorded to describe the environmental conditions during the trials (Fig. 1).



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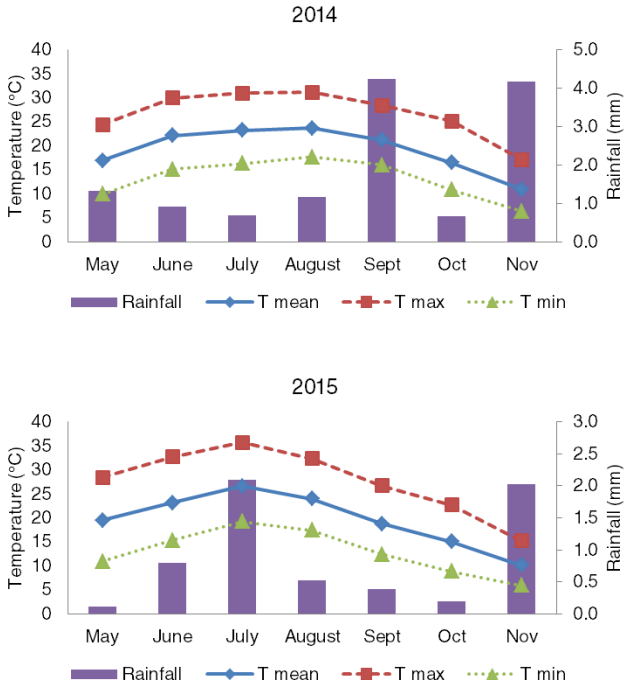

Figure 1. Meteorological data (temperature and rainfall) from May to November in the experimental field in Lleida (Catalonia, Spain) in 2013, 2014 and 2015.

2.4. Mycobiota determination

One hundred grains from each of the two maize varieties were randomly chosen immediately after harvest and decontaminated by immersion in 3% sodium hypochlorite (NaClO) (GPR Rectapur®, VWR Chemicals, Barcelona, Spain) followed by two rinses in sterile water. Kernels were plated in groups of five in dichloran rose-bengal chloramphenicol agar media (DRBC) Petri plates, which were then incubated for 7 days at 25 ºC in darkness. Colonies of developing fungi were isolated in potato dextrose agar (PDA), and the plates were incubated for 7 days at 25 ºC in darkness. Classification into genera was performed according to Pitt and Hocking (2009). The species level was identified using molecular biology techniques. The incidence of fungal infection was calculated as percentage considering that one hundred grains from each of the two maize varieties were isolated each harvest.

2.5. DNA extraction, amplification and identification

Pure cultures were grown in 500 µL malt extract broth in Eppendorf tubes (2% *w/v* malt extract, 1% *w/v* peptone, 2% *w/v* glucose) for 2 days at 25 °C in darkness. The mycelial extract was recovered by centrifugation (14,000 rpm, 10 min), and 300 µL of TNES buffer [200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% *w/v* sodium dodecyl sulfate (SDS)] was added. The mycelial suspension was lysed by vortexing with five 2.8 mm Precellys metal beads for 10 min. After centrifugation (14,000 rpm, 10 min), 150 µL of 3 mM sodium acetate (pH 5.2) was added to the supernatant. The supernatant was stored at -20 °C for 10 min and then centrifuged (14,000 rpm, 10 min). The DNA-containing supernatant was transferred to a new tube, and nucleic acids were precipitated by the addition of 1 volume of isopropanol. After a 5–min incubation at room temperature, the DNA suspension was centrifuged (14,000 rpm, 10 min). The DNA pellet was washed with 70% ethanol. The resulting pellet was air-dried, and the DNA was resuspended in 50 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). After extraction, the DNA concentrations were determined using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Firstly, all the isolates were subjected to amplification reactions for *Fusarium* identification. Secondly, amplification reactions for DNA sequencing were carried out with the isolates whose amplification product was negative for the specific *Fusarium* primers tested. Amplification reactions for *Fusarium* identification were conducted in volumes of 10 µL containing 10 ng template DNA, 0.5 µL of each primer (10 µM), 1 µl of 10x PCR buffer, 0.25 µL dNTPs (10 mM) and 0.1 µL Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Bioron GmbH, Ludwigshafen, Germany). The PCR buffer had the following composition 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween-20, 25 mM $MgCl_2$. To identify *Fusarium* isolates, specific primers were used (Table 1). Amplification reactions for DNA sequencing were conducted in volumes of 100 µL containing 10 ng template DNA, 4 µL of each primer (10 µM), 10 µL of 10x PCR buffer, 2 µL dNTPs (10 mM) and 0.75 µL Taq DNA polymerase (5 U/µL). The primers used for DNA sequencing are summarized in Table 2.

All PCR assays were performed using a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA) under the conditions summarized in Table 3. Amplification products were detected by

electrophoresis on 1.5% agarose ethidium-bromide gels in TAE 1x Buffer (40 mM Tris-acetate and 1 mM EDTA). A TrackIt™ 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular size marker. For DNA sequencing, PCR products were cleaned with the GeneJet PCR purification kit (Thermo Scientific, Waltham, MA, USA). The purified PCR products were sequenced by the company Macrogen Europe (Amsterdam, Netherlands). Sequences were analyzed and aligned using the Blast® search tool (National Center for Biotechnology Information, Bethesda, MD, USA).

Table 1. Specific primers for *Fusarium* identification.

Target	Primer name	Sequence (5'–3')	Size (bp)	Source
F. verticillioides	VERT-1	GTCAGAATCCATGCCAGAACG	800	Patiño *et al.*, 2004
	VERT-2	CACCCGCAGCAATCCATCAG		
F. proliferatum	Fp3-F	CGGCCACCAGAGGATGTG	230	Jurado *et al.*,
	Fp4-R	CAACACGAATCGCTTCCTGAC		2006
F. culmorum	Fc01F	ATGGTGAACTCGTCGTGGC	570	Nicholson *et al.*,
	Fc01R	CCCTTCTTACGCCAATCTCG		1998
F. graminearum	Fg16F	CTCCGGATATGTTGCGTCAA	450	Nicholson *et al.*,
	Fg16R	GGTAGGTATCCGACATGGCAA		1998
F. sporotrichioides	AF330109CF	AAAAGCCCAAATTGCTGATG	332	Demeke *et al.*,
	AF330109CR	TGGCATGTTCATTGTCACCT		2005

Table 2. Primers used for DNA sequencing.

Primer name	Sequence (5'–3')	Source
EF1	ATGGGTAAGGARGACAAGAC	O'Donnell *et al.*, 1998
EF2	GGARGTACCAGTSATCATGTT	
BT2A	GGTAACCAAATCGGTGCTGCTTTC	Glass & Donaldson, 1995
BT2B	ACCCTCAGTGTAGTGACCCTTGGC	
ITS1	TCCGTAGGTGAACCTGCGG	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC	

Table 3. PCR conditions used.

PCR assay	Initial denaturation[a]	Denaturation[b]	Annealing[b]	Extension[b]	Final extension[a]	Source
F. verticillioides	94 °C/2 min	95 °C/35 s	64 °C/30 s	72 °C/2 min	72 °C/5 min	Patiño *et al.*, 2004
F. proliferatum	94 °C/5 min	95 °C/30 s	69 °C/45 s	72 °C/45 s	72 °C/10 min	Jurado *et al.*, 2006
F. culmorum, F. graminearum, F. sporotrichioides	95 °C/3 min	95 °C/30 s	62 °C/20 s	72 °C/45 s	72 °C/5 min	Demeke *et al.*, 2005
Elongation factor	94 °C/5 min	94 °C/30 s	53 °C/30 s	72 °C/45 s	72 °C/10 min	O'Donnell & Cigelnik, 1997
β-tubulin	94 °C/5 min	94 °C/30 s	60 °C/45 s	72 °C/1 min	72 °C/7 min	Glass & Donaldson, 1995
ITS	94 °C/5 min	94 °C/30 s	50 °C/30 s	72 °C/45 s	72 °C/10 min	White *et al.*, 1990

[a] Conducted in one cycle.

[b] Conducted in 35 cycles (*F. verticillioides*, *F. proliferatum*, Elongation factor, β-tubulin and ITS) and 38 cycles (*F. culmorum*, *F. graminearum* and *F. sporotrichioides*).

2.6. Extraction, detection and quantification of FBs and AFs by HPLC

For fumonisin analysis, each sample (10 g maize + 1 g of sodium chloride) was extracted with 50 mL of acetonitrile: methanol: water (25: 25: 50 *v/v/v*) by blending for 20 minutes. The extracts were filtered, and 10 mL of each filtrate was diluted with 40 mL of phosphate buffered saline (PBS) solution at pH 7.4. For aflatoxin analysis, each sample (5 g maize) was extracted with 15 mL of methanol: water (60: 40 *v/v*) by blending for 10 minutes. The extracts were filtered, and 2 mL of each filtrate was diluted with 14 mL of PBS solution. In both cases, diluted extracts were cleaned up using immunoaffinity columns, Fumoniprep® for FBs and Easi-Extract® aflatoxin for AFs (R-Biopharm AG, Darmstadt, Germany). The filtrates were passed through the column at a flow rate of 2 mL/min, and the columns were then washed by passing 20 mL of PBS through at a flow rate of 5 mL/min. Finally, the toxins were eluted using 3 mL of methanol slowly passed through the column, and the solvent was evaporated to dryness under a nitrogen stream. The dried samples were re-dissolved in 500 µL methanol: water (50: 50 *v/v*), and a volume of 200 µL was injected into the HPLC system.

FBs (FB_1 + FB_2) and AFs (AFB_1 + AFB_2 + AFG_1 + AFG_2) were detected and quantified separately using an HPLC system (Waters, Milford, MA, USA) and a C18 column (5 µm Waters Spherisorb, 4.6 x 250 mm ODS2). For fluorescence detection, a Waters 2475 module was used at the following wavelengths: λexc 362 nm and λem 440 nm for AFs; λexc 335 nm and λem 440 nm for FBs. FBs were manually derivatized with ortho-phthalaldehyde (OPA) (Sydenham *et al.*, 1996), while AFs were photochemically derivatized (UVE™, LC Tech GmbH, Dorfen, Germany). The analysis was performed under isocratic conditions at a flow rate of 1 mL/min, and the mobile phase used was water: acetonitrile: methanol (70: 17: 17) for AFs and methanol: 0.1 M sodium dihydrogen phosphate solution (77: 23) (adjusted to pH 3.35 with orthophosphoric acid) for FBs. The detection limits were established based on a signal-to-noise ratio of 3: 1 and were 0.25 ng/g for AFB_1 and AFG_1, 0.15 ng/g for AFB_2 and AFG_2, 0.02 µg/g for FB_1 and 0.04 µg/g for FB_2. Quantification was performed using a software integrator (Empower, Milford, MA, USA). The analytical methods were validated according to the Commission Regulation No. 401/2006 (European Commission, 2006a). The recovery percentage obtained was considered to express the results.

2.7. Statistical analysis

ANOVA statistical tests and t-test for means comparison were employed (JMP® Pro 12 SAS institute, 2015). Differences among means with $p < 0.05$ were accepted as representing statistically significant differences.

3. Results

3.1. Moisture and water activity

The water activity and moisture values of the field samples were similar for both types of maize, HC and its near isogenic line (Table 4). However, the moisture content was higher during the third year of study in both types of maize, probably due to the delayed sowing time.

Table 4. Water activity (a_w) and moisture content (m.c., in %) after harvest in control and high-carotenoid maize during the trials. Means within a column with no superscript in common are significantly different ($p < 0.05$).

	2013		2014		2015	
	a_w	m.c.	a_w	m.c.	a_w	m.c.
Control maize	0.893 [b]	23.15 [b]	0.954 [a]	30.13 [a]	0.968 [a]	41.59 [a]
High-carotenoid maize	0.921 [a]	26.83 [a]	0.954 [a]	27.20 [b]	0.972 [a]	42.06 [a]

3.2. Mycobiota incidence and identification

The incidence of fungal infection was the same for both types of maize in 2013 (90%). In 2014, it was 86 and 81% for control and HC maize, respectively. In 2015, it was 100 and 86% for control and HC maize, respectively. Thus, the percentage of infected grains was 5% and 14% higher in control maize than in HC maize in 2014 and 2015, respectively.

Fusarium was the dominant genus isolated, present in more than 60% of both types of maize in all years of the study. The most common species isolated within this genus was *F. verticillioides*, although there was a decrease in its presence in favor of *F. proliferatum* in control maize in 2014. Other *Fusarium* spp., such as *Fusarium subglutinans* and *Fusarium poae,* were occasionally isolated. Other less frequently isolated fungi were *Acremonium strictum*, *Chaetomium globosum*, *Epicocum nigrum* and *Lecanicillium muscarium,* and other occasionally isolated genera were *Cladosporium*, *Penicillium, Alternaria*, *Talaromyces*, *Galactomyces* and *Sarocladium*. Nonetheless, all of these fungi

accounted for less than 15% of total infection in both types of maize in all the years of study, except for HC maize in 2013, in which the presence of other fungi increased to 30%, *A. strictum* the fungus responsible for half of this contamination. There was no *Aspergillus* spp. presence in any of the years studied. Figure 2 shows the identified mycobiota in both types of maize during the trials.

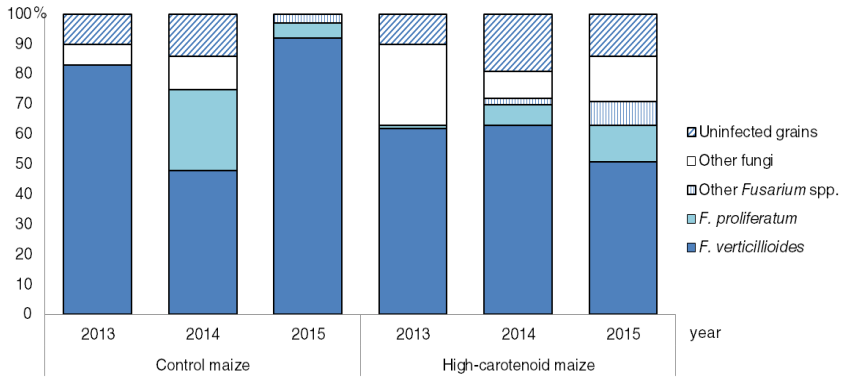

Figure 2. Mycobiota identification in control and high-carotenoid maize during the trials.

3.3. Fumonisin and aflatoxin contamination

Both types of maize were contaminated with FBs in all three years of the study, but control maize was 1.4–, 2.4– and 2–fold more contaminated than HC maize in 2013, 2014 and 2015, respectively. This difference was statistically significant ($p < 0.05$) in 2014 and 2015 (Fig. 3). On the other hand, there was no contamination by AFs in any of the years.

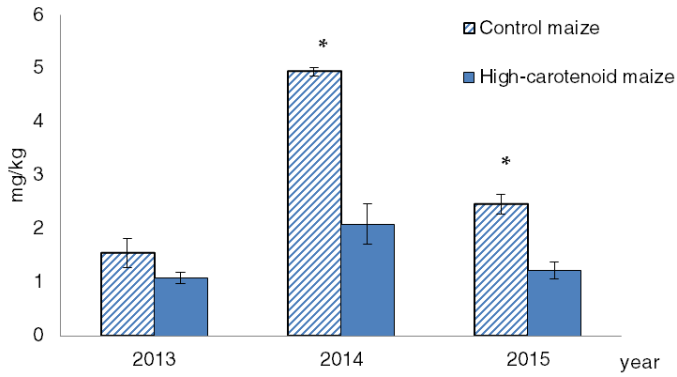

Figure 3. Fumonisin levels (FB_1 + FB_2, in mg/kg) in control and high-carotenoid maize during the trials. The values shown are the mean and standard error for each treatment (n = 3). The asterisk indicates statistically significant differences between types of maize ($p < 0.05$).

4. Discussion

HC maize can be a sustainable and cost-effective alternative to vitamin A supplementation in developing countries, as it can provide the recommended daily intake of PVA in 200 g of grain (Naqvi *et al.*, 2009). Its vulnerability to fungal infection and mycotoxin contamination compared to its near isogenic line has been evaluated in open field trials.

This study has shown slight differences in fungal infection between types of maize, with *F. verticillioides* being the most common species isolated in all three harvest seasons. Our results are consistent with previous studies that established that *F. verticillioides* and *F. proliferatum* are the most prevalent fungi in maize in our area (Marín *et al.*, 2012), although Bakan *et al.* (2002) found that *F. proliferatum* was more common than *F. verticillioides* in other fields located in Northeastern Spain. Fumonisin contamination was detected in all three years of the study, agreeing with previous studies that reported fumonisin contamination in maize products in the Spanish market (Velluti *et al.*, 2001; Cano-Sancho *et al.*, 2012). Considering the results obtained in our experiments, only the control maize harvested in 2014 had fumonisin levels above the maximum allowed level established by the European legislation for unprocessed maize (4000 μg/kg) (European Commission, 2006b, 2007). In 2014, there was higher fumonisin contamination in both types of maize compared to the other harvest seasons that could be explained by the

environmental conditions, as high rainfall was recorded two months before harvest in 2014, whereas the climatic conditions in 2013 and 2015 were quite similar (Fig. 1). This observation is in accordance with Cao *et al.* (2014) which pointed out that fumonisin accumulation is favored by hard rainfall during kernel drying and Marín *et al.* (2004) which reviewed the ecophysiology of fumonisin-producing *Fusarium* species and reported that during the ripening and final drying in the field, there are many opportunities for FB_1 production.

The differences in fumonisin contamination between types of maize were significant in two of the three years of the study and could be related to their differences in carotenoid content. Both types of maize had the same genetic background, as control maize was the basis for creating the HC maize. Thus, the only difference between them was the carotenoid content which was 88 ± 8 μg/g dw (dry weight) for HC maize and 1 ± 0.5 μg/g dw for control maize. Zeaxanthin levels were 23 and 0.3 μg/g dw, and lutein levels were 9 and 0.6 μg/g dw for HC and control maize, respectively. PVA carotenoids were only detected in HC maize (5.9 ± 2 and 3.7 ± 0.6 μg/g dw for β-carotene and β-cryptoxanthin, respectively) (Zanga *et al.*, personal communication).

Carotenoids are well-known antioxidants (Stahl and Sies, 2003), although the effect of carotenoids on mycotoxin biosynthesis has been scarcely studied. Fungi are often exposed to unfavorable environmental conditions, such as oxidative stress. It has been suggested that oxidative stress may be related to mycotoxin production, some toxins may be produced to counteract the excessive accumulation of reactive oxygen species (Reverberi *et al.*, 2010). Thus, as fumonisin biosynthesis can be stimulated by oxidative stress, the presence of antioxidants might therefore help to disrupt toxin accumulation (Picot *et al.*, 2010). This is supported by a recent review which has highlighted that antioxidant compounds such as carotenoids can quench oxygen free radicals produced by plant cells as a defense response, contributing to reduce oxidative stress that modulates toxin biosynthesis. Moreover, these lipophilic secondary metabolites can scavenge lipid peroxyl free radicals, reducing the lipid peroxidation (Atanasova-Pénichon *et al.*, 2016).

Regarding FBs, Picot *et al.* (2013) studied the potential involvement of maize antioxidants (ferulic acid, tocopherols and carotenoids) in resistance to *Fusarium* ear rot and fumonisin accumulation. Lutein (0.4 μg/mL),

zeaxanthin (0.4 μg/mL) and β-carotene (0.2 μg/mL) activity levels were evaluated *in vitro*. In lutein-supplemented liquid cultures, a reduction (52%) was observed in fumonisin production by one of the three strains of *F. verticillioides* tested, but the difference was not significant compared to the control treatments. Delgado *et al.* (2014) found a statistically significant positive correlation between lutein content and *Fusarium* mycotoxins in durum wheat, but FBs were not evaluated. With respect to AFs, it has been reported that the presence of carotenoids may reduce aflatoxin biosynthesis by *A. flavus* (Norton, 1997; Wicklow *et al.*, 1998).

Many factors can modulate fumonisin biosynthesis: genes such as the FUM gene cluster, ecophysiological factors such as water activity and temperature, physicochemical and nutritional factors such as pH and C:N ratio, and plant defense metabolites (Picot *et al.*, 2010). Endogenous plant compounds, both constitutive and induced in response to pathogen infection, may disrupt toxin biosynthesis (Boutigny *et al.*, 2008). It has been reported that the physiological stages of the maize kernel play a role in the regulation of fumonisin biosynthesis. Fungal infection can occur from the blister stage, but fumonisin production is not initiated until the dough stage. It has been shown that the dent stage can increase fumonisin biosynthesis (Picot *et al.*, 2011). Considering these previous results, the presence of carotenoids has been demonstrated in maize kernels from the blister to the final mature stages. Therefore, carotenoids are expected to be present when fumonisin biosynthesis occurs (Picot *et al.*, 2013).

The carotenoid enhancement in transgenic lines has been shown to affect starch metabolism. In transgenic lines with a total carotenoid content similar to the HC maize, the total starch content was found to decrease by up to 8% compared to the isogenic maize (Berman, 2016). Many hypotheses have been formulated regarding the role of the endosperm composition in fumonisin biosynthesis (Santiago *et al.*, 2015). Bluhm and Woloshuk (2005) noted that amylopectin presence might induce fumonisin production by *F. verticillioides*. Blandino and Reyneri (2007) studied *waxy* maize, which produces an endosperm granule that contains almost 100% amylopectin, showing that the waxy hybrids were more susceptible to fumonisin accumulation than the near isogenic lines. However, Picot *et al.* (2011) suggested that under field conditions, amylopectin is not the only component that favors fumonisin biosynthesis. The increase in fumonisin production appeared three weeks after the completion of the kernel with amylopectin, suggesting that other mechanisms such as pH may be involved. Carotenoid-enriched maize lines

had a slight difference in starch content compared to their near isogenic line. It would be interesting to study in detail whether starch content and composition in carotenoid-enriched maize lines could play a role in fumonisin reduction.

Carotenoids, as potent maize antioxidant compounds, may disrupt fumonisin biosynthesis or accumulation in maize plants. It has been demonstrated that the HC maize not only does not differ from its near isogenic line in fungal infection but also has a positive effect in terms of fumonisin reduction. Nonetheless, it is important to bear in mind that carotenoids may play a role together or individually. Further studies are necessary to clarify the effect of carotenoids on fumonisin production.

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CHAPTER II

High-carotenoid biofortified maize is an alternative to color additives in poultry feed

Joana Díaz-Gómez, Jose A. Moreno, Eduardo Angulo, Gerhard Sandmann, Changfu Zhu, Antonio J. Ramos, Teresa Capell, Paul Christou and Carmina Nogareda

Animal Feed Science and Technology (2017),

231: 38–46

High-carotenoid biofortified maize is an alternative to color additives in poultry feed

Joana Díaz-Gómez [a, b], José A. Moreno [b], Eduardo Angulo [b], Gerhard Sandmann [c], Changfu Zhu [d], Antonio J. Ramos [a], Teresa Capell [d], Paul Christou [d, e] and Carmina Nogareda [b]

[a] Food Technology Department, University of Lleida–Agrotecnio Center; [b] Animal Science Department; [c] Biosynthesis Group, Department of Molecular Biosciences, J. W. Goethe University, Max von Laue Str. 9, D-60438 Frankfurt, Germany; [d] Plant Production and Forestry Science Department; [a, b, d] University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain; [e] ICREA, Catalan Institute for Research and Advanced Studies, Pg. Lluís Companys 23, 08010 Barcelona, Spain

Abstract

Skin color in poultry is achieved by the addition of natural or synthetic pigments to feed. Crops used routinely in feed formulations offer an alternative cost-effective strategy to replace color additives if they are biofortified with sufficient levels of carotenoids. We tested the hypothesis that high-carotenoid (HC) maize, which was genetically engineered to accumulate high levels of β-carotene, lutein and zeaxanthin in the endosperm, can replace carotenoid additives in poultry feed by performing two feeding trials using diets incorporating different maize lines with diverse carotenoid compositions: control (wild-type M37W, the parental line), HC, and standard yellow commercial maize supplemented with color additives (marigold flowers and red paprika extracts). The effects of dietary treatments on growth performance, health parameters, color evolution and carotenoid distribution were determined. We found that chickens fed on the HC diet grew normally and developed similar pigmentation to animals fed on a commercial diet supplemented with color additives, although yellowness was significantly higher in the commercial diet due to the high concentration of yellow xanthophylls. Lightness scores in chickens fed on the control, HC and commercial diets were 45.88 ± 1.31, 44.32 ± 1.10 and 44.29 ± 0.99, respectively, in breast muscle, and 51.62 ± 1.33, 49.66 ± 0.96 and 50.10 ± 1.16, respectively, in thigh muscle. Redness scores in chickens fed on the control, HC, and commercial diets were 0.36 ± 0.26, 3.25 ± 0.29 and 3.58 ± 0.29, respectively, in breast muscle, and 1.28 ± 0.37, 4.79 ± 0.39 and 4.85 ±

0.34, respectively, in thigh muscle. Yellowness scores in chickens fed on the control, HC, and commercial diets were 2.45 ± 0.47, 7.61 ± 0.64 and 9.66 ± 0.73, respectively, in breast muscle, and 3.38 ± 0.64, 10.00 ± 1.10 and 12.64 ± 0.97, respectively, in thigh muscle. High-carotenoid maize is therefore a cost-effective alternative to feed supplementation in the poultry industry.

Keywords

Chicken, biofortification, carotenoids, metabolic engineering, pigments

1. Introduction

Skin color is the first quality attribute of poultry meat that is evaluated by consumers. A golden skin color is preferred by consumers, especially in North America and the Asia-Pacific markets, because this is associated with a normal state of health (Williams, 1992). Skin pigmentation is affected by genotype, the quantity and dietary source of pigments, and the health of the birds, among other factors (Sirri *et al.*, 2010). In poultry meat, skin color is provided by carotenoid pigments present in the feed that are deposited in the skin and subcutaneous fat (Pérez-Vendrell *et al.*, 2001). Both natural and synthetic pigments can be used as feed additives in the poultry industry to achieve the level of skin pigmentation desired by consumers, but this increases the production costs (Castañeda *et al.*, 2005; Tarique *et al.*, 2013).

Carotenoids are ubiquitous isoprenoid-based natural pigments that can be classed as carotenes, which contain only hydrogen and carbon, or xanthophylls, which also contain oxygen (Britton, 1995). Some carotenoids have provitamin A (PVA) activity, including α-carotene, β-carotene and β-cryptoxanthin, whereas others have no PVA activity but can act as antioxidants (Farré *et al.*, 2010). Carotenoids improve disease resistance in birds and mammals (Surai, 2002) and can also modulate the immune system (Chew and Park, 2004). Chickens, like most other animals, cannot synthesize carotenoids *de novo* and must obtain them from their diet (Breithaupt, 2007; Nogareda *et al.*, 2016). Vitamin A and carotenoid metabolism in chickens is closely related to the equivalent processes in humans, so chickens are also susceptible to vitamin A deficiency with similar symptoms (NRC, 1994; Pretorius and Schönfeldt, 2013).

The value of the global carotenoids market was an estimated $US 1.5 billion in 2014, and is expected to reach nearly $US 1.8 billion in 2019 (BCC Research, 2015). This increasing demand for dietary pigments in the

food/feed industry makes it necessary to find cost-effective alternatives. Novel strategies have been developed recently to enhance carotenoid levels in staple crops (Bai *et al.*, 2011; Farré *et al.*, 2014). The South African white-endosperm maize inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase 1 (PSY1), has been used as a basis for the development of high-carotenoid (HC) maize, which expresses PSY1 and the bacterial enzyme carotene desaturase (CrtI) and therefore accumulates high levels of β-carotene, lutein and zeaxanthin (Zhu *et al.*, 2008).

To address our hypothesis that HC maize is a suitable replacement for carotenoid additives in poultry feed, we performed two animal feeding trials to evaluate three diets containing different types of maize: control (wild-type M37W), HC, and standard yellow commercial maize supplemented with color additives (marigold flowers and red paprika extracts). Chickens fed on these diets were compared in terms of productivity and health parameters, including coloration and carotenoid distribution.

2. Materials and methods

2.1. Diet preparation

The South African white maize inbred M37W and its engineered derivative HC (Zhu *et al.*, 2008) were grown in an experimental field in Northeastern Spain (Lleida, Catalonia) for two consecutive seasons. They were used to prepare the diets together with standard yellow commercial maize provided by the feed industry. The diets were prepared at the Mas de Bover Research Center (IRTA, Reus, Spain) and formulated according to National Research Council (NRC) recommendations (NRC, 1994).

Maize-supplemented diets were prepared by mixing ~50 kg of milled maize with a commercial poultry diet and adjusting the other ingredients to maintain nutritional balance (Table 1). Vitamin A is included to ensure normal growth and development, and was present in all the diets: 10,000 IU/kg (3 mg retinol/kg) for starter diets and 8,000 IU/kg (2.4 mg retinol/kg) for grower diets (Yuan *et al.*, 2014). Three diets were prepared: control diet (M37W maize), high-carotenoid diet (HC maize), and commercial diet supplemented with color additives (standard yellow maize). The control diet was prepared before the carotenoid-enriched diets to prevent cross-contamination. Taking into account the pigment characteristics

of the standard yellow commercial maize, the quantity of color additives was calculated using standard industry methods (Amaya *et al.*, 2014). The commercial diet was therefore supplemented with 31 mg/kg yellow xanthophylls (marigold flower extract; Capsantal EBS-40-NT) and 3 mg/kg of red xanthophylls (red paprika extract; Capsantal FS-20-NT), both provided by Industrial Técnica Pecuaria S.A. (Barcelona, Spain). The humidity, protein, fat, fiber and ash content of the blended maize grains and formulated diets was measured according to European Commission Regulation No. 152/2009 (European Commission, 2009). Mycotoxin levels were determined using enzyme immunoassay kits (Ridascreen®, R-Biopharm AG, Darmstadt, Germany).

Table 1. Composition of starter (days 0–9) and grower (days 10–35) diets and chemical analysis of the grower diets. HC, high-carotenoid.

Ingredient (g/kg)	Starter control	Starter HC	Starter commercial	Grower control	Grower HC	Grower commercial
Control maize	522.00	0	0	582.00	0	0
High-carotenoid maize	0	522.00	0	0	582.00	0
Commercial maize	0	0	522.00	0	0	582.00
Soybean meal 47.5%	361.00	361.00	361.00	178.50	178.50	178.50
Soy hulls	0	0	0	158.50	158.50	158.50
Soybean oil	68.00	68.00	68.00	42.00	42.00	42.00
Monocalcium phosphate	15.23	15.23	15.23	12.28	12.28	12.28
Calcium carbonate	14.55	14.55	14.55	10.97	10.97	10.97
Sodium chloride	3.47	3.47	3.47	3.17	3.17	3.17
Sodium bicarbonate	2.33	2.33	2.33	1.19	1.19	1.19
Choline chloride 60%	0.51	0.51	0.51	0.42	0.42	0.42
L-Lysine chloride 79%	2.69	2.69	2.69	2.77	2.77	2.77
DL-Methionine 99%	3.26	3.26	3.26	3.33	3.33	3.33
L-Threonine 98%	0.42	0.42	0.42	0.47	0.47	0.47
Vitamin-mineral premix [1]	3.03	3.03	3.03	3.03	3.03	3.03
Vitamin premix starter [2]	3.03	3.03	3.03	0	0	0
Vitamin premix grower [3]	0	0	0	1.04	1.04	1.04
Coccidiostat (cygro)	0.51	0.51	0.51	0	0	0
Coccidiostat salinomycin 12%	0	0	0	0.52	0.52	0.52
Mixture of color additives	0	0	0	0	0	0.93

Chemical analysis (g/kg)	Starter control	Starter HC	Starter commercial	Grower control	Grower HC	Grower commercial
Crude protein	-	-	-	218.50	219.60	190.50
Crude fiber	-	-	-	45.30	40.80	41.00
Crude fat	-	-	-	99.20	99.40	91.70
Ash	-	-	-	48.10	57.40	54.60
Moisture	-	-	-	143.90	102.90	124.00

[1] Vitamin-mineral premix: vitamin D_3 1,700 IU/kg; vitamin B_1 2 mg/kg; vitamin B_2 6.4 mg/kg; vitamin B_6 3 mg/kg; vitamin B_{12} 0.02 mg/kg; vitamin E 50 mg/kg; vitamin K 3 mg/kg; folic acid 1 mg/kg; nicotinic acid 40 mg/kg; panthotenic acid 11.7 mg/kg; biotin 0.1 mg/kg; cooper 6 mg/kg; zinc 54 mg/kg; iron 40 mg/kg; manganese 77 mg/kg; selenium 0.45 mg/kg; iodine 2.28 mg/kg; BHT antioxidant 125 mg/kg.

[2] Vitamin premix starter: vitamin A 10,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

[3] Vitamin premix grower: vitamin A 8,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

2.2. Bird management and experimental design

In each trial, 1-day-old male broiler chickens of the Ross 308 strain purchased from a commercial hatchery were initially weighed, wing-banded for individual identification and randomly allocated into pens in the Animal Research Center of the University of Lleida. Six broilers were allocated to each of four pens per treatment, i.e. 24 broilers per treatment. Pens were separated by solid partitions to avoid cross-contamination caused by feed spreading. The broilers were presented with starter feed (52% maize, 36% soybean meal and 7% soybean oil) on days 0–8 and grower feed (58% maize, 18% soybean meal, 16% soybean hull and 4% soybean oil) on days 9–35. Feed and drinking water were provided *ad libitum*. No medication was administered during the feeding trial. Management parameters (temperature, humidity, lighting and ventilation) were monitored and changed according to the age of the chickens, following NRC guidelines (NRC, 2011).

Animals were handled according to Directive 2010/63/EU (European Parliament, 2010) and Commission Recommendation 2007/526/EC (European Commission, 2007), as well as the best practices recommended by the International Life Sciences Institute (ILSI, 2007). All experimental procedures were approved by the Ethics Committee for Animal

Experimentation of the University of Lleida and the Catalan Government (reference numbers DAAM 7672 and DAAM 7743).

2.3. Growth performance

Body weight and feed weight were determined every 7 days (and on day 9, when starter diet was changed to grower diet). Average daily gain (ADG), body weight gain, feed intake and feed conversion ratio (FCR) were calculated at the end of the trial. All chickens were humanely euthanized on day 35 and gross necropsy was carried out. Blood samples were randomly collected in three chickens of each pen for biochemical and hematological analysis, and serum was separated and frozen for carotenoid analysis. Pre-chilled whole liver, spleen and bursa of Fabricius were collected and weighed. Pre-chilled samples of liver were freeze-dried (fd) for carotenoid analysis.

2.4. Carotenoid analysis

Total carotenoids were extracted from freeze-dried samples in 20 mL methanol containing 12% KOH at 65 °C for 1 h. Lipophilic compounds were partitioned into 30% diethyl ether in petroleum ether, the upper phase was collected and the solvent was evaporated under a stream of nitrogen gas at 37 °C. For separation by high-performance liquid chromatography (HPLC), samples were re-dissolved in 100 μL methanol/dichloromethane (50: 50 *v*/*v*) and a 20-μL aliquot was injected immediately. Compounds were separated on a 15-cm Nucleosil C18 3-μm column with an acetonitrile, methanol and 2-propanol mobile phase (85: 15: 5 *v*/*v*/*v*) at 20 °C. Samples were monitored with a Kontron 440 photodiode array detector with online registration of the spectra and were identified by comparison against authentic reference compounds (Sandmann, 2002).

2.5. Colorimetric analysis

The color was measured using the CIELab trichromatic system (CIE, 2004) as lightness (L*), redness (a*) and yellowness (b*) with a CM-700d compact portable colorimeter (Konica Minolta, Tokyo, Japan). Coordinate L* represents lightness ranging from 0 (black) to 100 (white), a* indicates the red/green component and b* indicates the yellow/blue component. The range of both chromatic components is between −128 and 128 (Sharifzadeh *et al.*, 2014). The illuminant D65 and a 10° viewing angle were used for all measurements. The measuring area was changed according to the footpad

size, using a mask of 3-mm-diameter during the first week and a mask of 8-mm-diameter from then onward. During the feeding trial, the color was measured weekly in the footpad with prior cleaning if necessary. After slaughter, the color was measured in pre-chilled footpad, breast skin, and breast and thigh muscles.

2.6. Statistical analysis

Experimental data were analyzed using JMP® Pro 12 (SAS institute, 2015) and differences were considered significant at the 5% level of probability. The experimental unit was the pen, except for carotenoid levels, and the MIXED procedure was applied, including fixed effect of dietary treatments, feeding trial (as there were only two levels) and their interaction, and random effect of pens: $Y_{ijke} = Diet_i + Trial_j + Diet_i*Trial_j + Pen_k + \varepsilon_{ijke}$. For statistical analysis of carotenoids, analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test was used, and for color evaluation, pairwise correlations were used. Variables expressed in percentages were normalized using the arcsine of the square root of the probability.

3. Results

3.1. Compositional analysis

A standard compositional analysis for humidity, protein, fat, fiber and ash content revealed that the diets were not substantially different, although protein levels were slightly lower in the commercial diet (Table 1). Carotenoid analysis of the grower diets (used from days 9–35) showed that the HC diet had the highest levels of zeaxanthin (10.33 μg/g fd diet formulation), β-carotene (3.04 μg/g fd) and β-cryptoxanthin (2.09 μg/g fd), whereas the commercial diet supplemented with color additives had the highest lutein levels (23.69 μg/g fd) due to the presence of marigold extract (Table 2). Capsanthin was only detected in commercial diet supplemented with color additives due to the presence of paprika extract.

Table 2. Carotenoid composition (μg/g freeze-dried feed) of the grower diets (used on days 9–35) in the first and second animal feeding trials. Values are means ± standard errors (n = 3). Means within a row with no superscript in common are significantly different ($p < 0.05$). nd: not detected.

1st feeding trial	Control diet	High-carotenoid diet	Commercial diet + color additives
Violaxanthin	0.13 ± 0.00 c	2.77 ± 0.31 a	0.9 ± 0.08 b
Lutein	1.05 ± 0.01 c	3.69 ± 0.47 b	25.77 ± 0.96 a
Zeaxanthin	0.40 ± 0.01 c	10.85 ± 1.18 a	5.68 ± 0.42 b
β-cryptoxanthin	0.25 ± 0.01 c	3.15 ± 0.37 a	0.4 ± 0.03 c
β-carotene	0.07 ± 0.01 b	3.02 ± 0.31 a	traces
2nd feeding trial	**Control diet**	**High-carotenoid diet**	**Commercial diet + color additives**
Violaxanthin	nd	0.11 ± 0.01	nd
Lutein	1.18 ± 0.03 c	3.45 ± 0.12 b	21.61 ± 0.59 a
Zeaxanthin	1.55 ± 0.02 c	9.80 ± 0.58 a	4.99 ± 0.08 b
β-cryptoxanthin	nd	1.04 ± 0.06 a	0.84 ± 0.02 b
β-carotene	nd	3.06 ± 0.17 a	1.67 ± 0.00 b

3.2. Productivity and health parameters

According to the statistical analysis, there was no pen effect on any of the criteria evaluated. Chickens fed on the different diets had a similar body weight gain profile ($p > 0.05$) (Fig. 1). Despite there was no treatment effect, chickens from the first experiment were heavier than chickens from the second one. There were no significant differences ($p > 0.05$) in the overall body weight gain, ADG and feed intake whereas the FCR was significantly higher in the commercial diet ($p < 0.05$) (Table 3). The weight of the liver, spleen and bursa of Fabricius was similar among chickens fed on the three diets ($p > 0.05$) (Table 4).

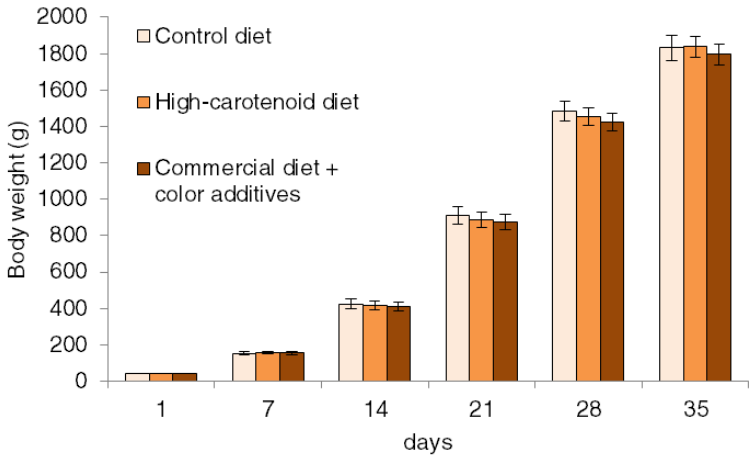

Figure 1. Body weight gain profile of chickens fed on the three diets for 5 weeks. Values are means ± standard errors (n = 8).

Table 3. Analysis of broiler production parameters: initial and final body weight (bw), average daily gain (ADG), feed intake and feed conversion ratio (FCR). Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Initial bw (g)	40.65 ± 1.12	40.84 ± 1.01	41.11 ± 1.14
Final bw (g)	1831.35 ± 69.26	1837.31 ± 56.13	1796.76 ± 57.12
ADG 1st week (g)	15.84 ± 1.21	16.44 ± 0.82	16.12 ± 1.02
ADG 2nd week (g)	38.91 ± 3.10	37.27 ± 2.81	36.59 ± 2.42
ADG 3rd week (g)	69.34 ± 3.60	67.09 ± 3.27	66.30 ± 3.68
ADG 4th week (g)	82.10 ± 4.22	80.75 ± 4.57	78.53 ± 4.40
ADG 5th week (g)	49.62 ± 4.31	55.10 ± 4.03	53.27 ± 4.70
ADG final (g)	51.16 ± 1.97	51.33 ± 1.60	50.16 ± 1.62
Feed intake (g)	2918.17 ± 90.99	2849.02 ± 29.19	2999.77 ± 93.86
FCR	1.63 ± 0.02 [a, b]	1.59 ± 0.01 [b]	1.67 ± 0.02 [a]

Table 4. Effects of feed diets on the relative organ weight of broilers (expressed as % relative to whole live animal weight). Values are means ± standard errors (n = 8).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Liver	1.95 ± 0.10	2.02 ± 0.12	2.08 ± 0.10
Spleen	0.13 ± 0.02	0.13 ± 0.02	0.14 ± 0.01
Bursa of Fabricius	0.16 ± 0.03	0.15 ± 0.02	0.16 ± 0.03

The analysis of biochemical parameters revealed that bilirubin levels were significantly higher ($p < 0.05$) in chickens fed on the HC and commercial diets compared to those fed on the control diet. Creatinine levels were significantly lower ($p < 0.05$) in chickens fed on the HC diet compared to those fed on the control diet whereas animals fed on the commercial diet had significantly lower ($p < 0.05$) albumin levels compared to those fed on the control diet (Table 5). The analysis of hematological parameters indicated that the commercial diet resulted in a significantly higher ($p < 0.05$) lymphocyte count than the HC diet group, although the proportion of lymphocytes did not differ significantly among the three diet groups ($p > 0.05$) (Table 6).

Table 5. Biochemical values after slaughter measured in blood samples from chickens fed on the three diets. Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Glucose (mg/dL)	228.38 ± 6.02	225.81 ± 5.49	225.90 ± 5.85
Calcium (mg/dL)	102.76 ± 1.96	102.71 ± 1.86	103.28 ± 1.95
Total protein (g/dL)	33.17 ± 1.18	32.77 ± 1.01	33.21 ± 1.21
Aspartate transaminase (IU/L)	295.90 ± 43.76	255.45 ± 14.76	248.69 ± 14.92
Total Bilirubin (mg/dL)	0.11 ± 0.02 [b]	0.18 ± 0.02 [a]	0.20 ± 0.02 [a]
Potassium (mEq/L)	5.85 ± 0.58	5.88 ± 0.41	5.68 ± 0.36
Creatinine (g/dL)	0.25 ± 0.01 [a]	0.23 ± 0.01 [b]	0.24 ± 0.01 [a, b]
Phosphorus (mg/dL)	8.02 ± 0.38	8.00 ± 0.24	7.74 ± 0.21
Albumin (mg/dL)	12.17 ± 0.33 [a]	11.74 ± 0.43 [a, b]	11.55 ± 0.29 [b]
Alanine transaminase (IU/L)	2.55 ± 0.61	2.52 ± 0.34	2.38 ± 0.22
Lactate dehydrogenase (IU/L)	3896.52 ± 627.96	3255.06 ± 371.85	2881.38 ± 367.00
Uric acid (mg/dL)	4.34 ± 0.51	4.93 ± 0.98	3.74 ± 0.63
Total Cholesterol (mg/dL)	162.34 ± 5.86	164.77 ± 6.86	165.00 ± 6.97
Alkaline phosphatase (IU/L)	4884.24 ± 969.88	5123.55 ± 1065.15	5316.83 ± 1424.52
Gamma-glutamyl transpeptidase (IU/L)	25.83 ± 1.41	25.42 ± 1.83	24.03 ± 1.82
Sodium (mEq/L)	152.17 ± 1.01	152.68 ± 0.92	151.66 ± 0.76
VLDL (mg/dL)	8.59 ± 1.16	8.84 ± 0.69	10.34 ± 2.05
HDL Cholesterol (mg/dL)	108.31 ± 3.47	105.10 ± 4.42	108.59 ± 4.23
LDL Cholesterol (mg/dL)	60.23 ± 6.89	63.81 ± 7.23	59.25 ± 6.90

Table 6. Hematological values after slaughter measured in blood samples from chickens fed on the three diets. Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$). The H/L ratio was calculated from the mean values of the heterophils and lymphocytes.

	Control diet	High-carotenoid diet	Commercial diet + color additives
Hematocrit (%)	0.38 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
Hemoglobin g/dL	12.47 ± 0.34	12.53 ± 0.40	12.04 ± 0.36
Total leukocytes/μL	13800.00 ± 2119.26	14216.67 ± 1866.95	17378.95 ± 3383.96
Eosinophils (%)	4.58 ± 1.37	4.96 ± 1.06	5.26 ± 1.12
Basophils (%)	9.47 ± 1.60	10.83 ± 2.47	9.79 ± 2.15
Lymphocytes (%)	39.53 ± 5.00	33.71 ± 3.55	40.89 ± 5.02
Monocytes (%)	8.16 ± 1.56	10.38 ± 2.26	7.32 ± 1.98
Heterophils (%)	36.37 ± 3.52	40.13 ± 3.51	36.74 ± 4.47
Eosinophils/μL	649.58 ± 259.69	754.08 ± 221.84	966.63 ± 324.11
Basophils/μL	1422.21 ± 375.01	1633.92 ± 479.15	1675.74 ± 477.68
Lymphocytes/μL	5493.63 ± 894.69 [a, b]	4658.46 ± 636.71 [b]	6860.84 ± 1448.54 [a]
Monocytes/μL	1111.16 ± 304.45	1357.42 ± 294.57	1268.63 ± 418.28
Heterophils/μL	5039.21 ± 898.30	5812.79 ± 1063.36	6607.11 ± 1694.76
H/L (1/1)	1.04 ± 0.27	1.39 ± 0.29	1.16 ± 0.35

3.3. Carotenoid and retinol levels

Carotenoid results reported here belong to the second animal feeding trial. Chickens fed on the HC diet had the highest retinol levels in liver, whereas chickens fed on the commercial diet supplemented with color additives had the highest retinol levels in the serum (Fig. 2). Nevertheless, these differences were only statistically significant in the liver ($p < 0.05$). Chickens fed on the HC diet had significantly higher levels of zeaxanthin ($p < 0.05$) and β-cryptoxanthin ($p < 0.001$) in the liver compared to the other diets, whereas chickens fed on the commercial diet supplemented with color additives had significantly higher levels of lutein ($p < 0.001$). The levels of zeaxanthin in the livers of chickens fed on the control and HC diets were 1.30 ± 0.05 and 7.19 ± 1.22 μg/g fd, respectively. The levels of lutein in the livers of chickens fed on the control, HC and commercial diets were 0.61 ± 0.02, 1.76 ± 0.26 and 15.40 ± 1.44 μg/g fd, respectively. Finally, the levels of β-cryptoxanthin in the livers of chickens fed on the HC and commercial diets were 7.42 ± 0.51 and 0.26 ± 0.05 μg/g fd, respectively. Zeaxanthin was

not detected in chickens fed on the commercial diet and β-cryptoxanthin was not detected in chickens fed on the control diet.

We did not detect any β-carotene in the liver or serum of any chickens in any of the diet groups. There were no significant differences in the levels of lutein in serum from chickens fed on any of the diets ($p > 0.05$). The serum lutein levels were 0.11 ± 0.00 μg/mL in chickens fed on the control and HC diets and 0.29 ± 0.11 μg/mL in chickens fed on the commercial diet. There were no significant differences in the levels of zeaxanthin ($p > 0.05$) between chickens fed on the HC (0.19 ± 0.00 μg/mL) and commercial (0.2 ± 0.01 μg/mL) diets whereas zeaxanthin was not detected in serum from chickens fed on the control diet. β-Cryptoxanthin was not detected in the serum of any chickens in any of the diet groups.

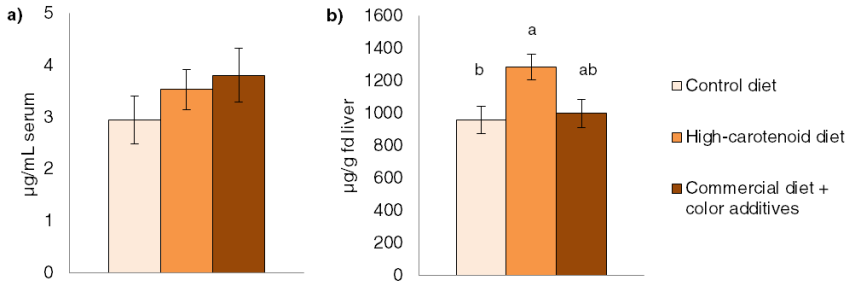

Figure 2. Retinol levels in serum (μg/mL) and liver (μg/g freeze-dried –fd–) measured in the second feeding trial in chickens fed on the three diets. Values are means ± standard errors. **a)** Serum (n = 6); **b)** liver (n = 8). Means within each graph with no superscript in common are significantly different ($p < 0.05$).

3.4. Color parameters

Skin color evolution was evaluated every week using the CIELab trichromatic system according to lightness (L*), redness (a*) and yellowness (b*). There were already significant differences ($p < 0.001$) in the redness and yellowness parameters 1 week into the feeding trial (Fig. 3). There was a continuous increase in those parameters in chickens fed on both the HC and commercial diets. The footpad color of chickens fed on the HC diet had a significantly lower lightness value ($p < 0.05$) than the control diet, but the footpad color of chickens fed on both the HC and commercial diets had significantly higher redness ($p < 0.001$) and yellowness ($p < 0.001$) values

than chickens fed on the control diet. After slaughter, these results were corroborated by measuring the color in footpad, breast skin, and breast and thigh muscles (Table 7). Chickens fed on the HC and commercial diets showed the highest redness values in breast and thigh muscles ($p < 0.001$), whereas the highest yellowness values ($p < 0.001$) were observed in chickens fed on the commercial diet supplemented with color additives.

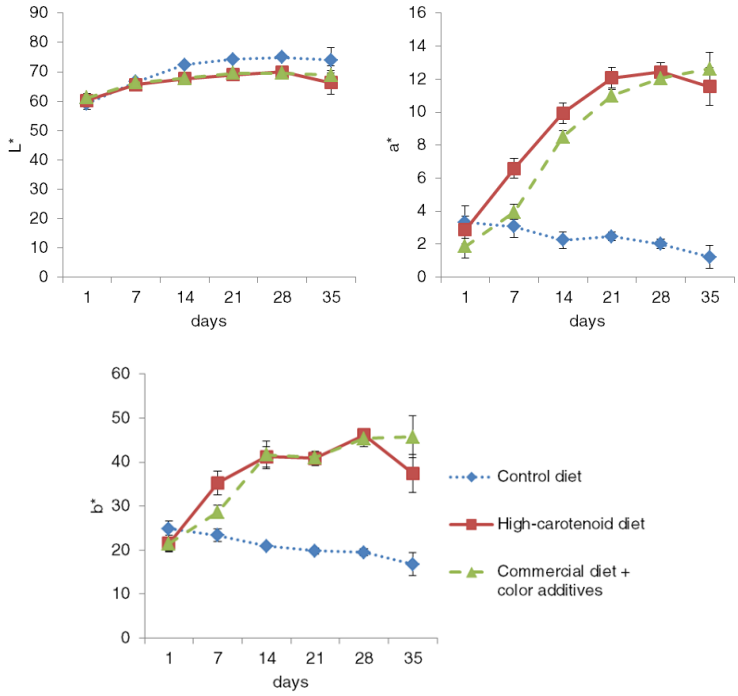

Figure 3. Color development measured in the footpad of chickens fed on the three diets for 5 weeks, using the CIELab trichromatic system to determine lightness (L*), redness (a*) and yellowness (b*). Values are means ± standard errors (n = 8).

There was a correlation in yellowness between breast and thigh muscles in chickens fed on all diet groups: control ($R^2 = 0.09$, $p < 0.05$), HC ($R^2 = 0.10$, $p < 0.05$) and commercial ($R^2 = 0.14$, $p < 0.05$) diets. There was also a correlation in yellowness between breast skin and breast muscle in chickens fed on the HC ($R^2 = 0.129$, $p < 0.05$) and commercial ($R^2 = 0.109$, $p < 0.05$) diets but not in those fed on the control diet ($p > 0.05$). There was no correlation between the breast and thigh muscles in terms of lightness or redness in any of the diet groups ($p > 0.05$). There was also no correlation in

terms of redness between breast skin and breast muscle in any of the diet groups ($p > 0.05$), and a correlation in lightness was only found in chickens fed on the control diet ($R^2 = 0.09$, $p < 0.05$). The color differences among chickens fed on the different diets are summarized in Figure 4.

Table 7. Color determination after slaughter in footpad, breast skin, breast muscle and thigh muscle of chickens fed on the three diets. Color determined using the CIELab trichromatic system as lightness (L*), redness (a*) and yellowness (b*). Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

		Control diet	High-carotenoid diet	Commercial diet + color additives
Footpad	L*	73.94 ± 4.31 ^{a}	66.36 ± 4.15 ^{b}	68.82 ± 3.27 ^{a,b}
	a*	1.22 ± 0.69 ^{b}	11.55 ± 1.14 ^{a}	12.63 ± 1.00 ^{a}
	b*	16.77 ± 2.61 ^{b}	37.48 ± 4.30 ^{a}	45.75 ± 4.75 ^{a}
Breast skin	L*	64.03 ± 1.55 ^{a}	60.85 ± 1.55 ^{b}	61.88 ± 1.62 ^{a,b}
	a*	0.01 ± 0.60 ^{b}	1.91 ± 0.70 ^{a}	2.10 ± 0.85 ^{a}
	b*	0.69 ± 1.43 ^{c}	8.60 ± 2.56 ^{b}	13.55 ± 2.06 ^{a}
Breast muscle	L*	45.88 ± 1.31	44.32 ± 1.10	44.29 ± 0.99
	a*	0.36 ± 0.26 ^{b}	3.25 ± 0.29 ^{a}	3.58 ± 0.29 ^{a}
	b*	2.45 ± 0.47 ^{c}	7.61 ± 0.64 ^{b}	9.66 ± 0.73 ^{a}
Thigh muscle	L*	51.62 ± 1.33 ^{a}	49.66 ± 0.96 ^{b}	50.10 ± 1.16 ^{b}
	a*	1.28 ± 0.37 ^{b}	4.79 ± 0.39 ^{a}	4.85 ± 0.34 ^{a}
	b*	3.38 ± 0.64 ^{c}	10.00 ± 1.10 ^{b}	12.64 ± 0.97 ^{a}

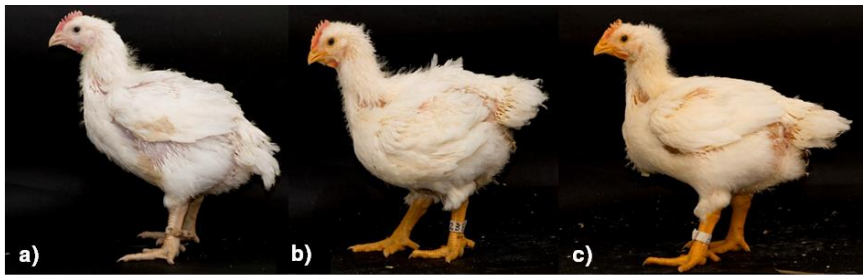

Figure 4. Four-week-old broilers fed on the three diets: **a)** Control diet; **b)** High-carotenoid diet; **c)** Commercial diet supplemented with color additives.

4. Discussion

Chickens, like most other animals, must obtain carotenoids from their diet because they cannot synthesize them naturally (Breithaupt, 2007). However, the crops typically used in commercial poultry feed (e.g. maize and soybean) do not supply sufficient carotenoids to achieve the skin pigmentation desired by many consumers in North America and the Asia-Pacific markets, and synthetic or natural carotenoids are therefore routinely added to feed formulations (Castañeda *et al.*, 2005; Williams, 1992). Larger quantities of color additives are required for today's poultry genotypes because they have been bred to grow rapidly, and this increases the cost of feed. Novel crops such as HC maize, which accumulates high levels of β-carotene, lutein and zeaxanthin, could therefore provide an alternative to color additives in the poultry industry. We tested the impact of a poultry diet based on HC maize by measuring its impact on productivity and health parameters, color development and the distribution of carotenoids compared to equivalent diets based on the near isogenic wild-type maize inbred M37W (which was used as the background to create the transgenic HC variety used in this study) and a commercial maize diet supplemented with color additives.

There were no significant differences in productivity parameters when we compared chickens fed on the control and HC diets. The FCR, which is defined as the weight of feed in kg required to produce a weight gain of 1 kg in a living animal, was significantly lower in the HC diet than in the commercial diet supplemented with color additives. The similar performance of the HC and control diets suggests that efficient FCR may be a property specific to the genetic background of these lines (M37W maize, the control maize, used as a basis for the development of HC) rather than the carotenoid content, given that the commercial diet is based on a mixture of yellow maize varieties and that feed supplementation with pigments does not appear to influence feed consumption or body weight (Liu *et al.*, 2008; Pérez-Vendrell *et al.*, 2001). Previous experiments have shown that broilers fed on the HC diet had a heavier bursa of Fabricius than chickens fed on the control diet which may reflect their better immunomodulatory response to vaccination against infectious bursal disease (Gumboro) (Nogareda *et al.*, 2016). However, we observed no statistically significant difference in the weight of this organ among chickens fed on the control, HC and commercial diets most likely because unlike the earlier experiments, chickens were not vaccinated in these trials. Bilirubin levels were higher in the chickens fed on the carotenoid-enriched diets and highest in those fed on the commercial

diet, which may reflect the onset of subclinical inflammation. Both bilirubin and carotenoids have immunomodulatory effects and this may explain the differences in lymphocytes levels in chickens fed on the three different diets (Koutsos *et al.*, 2003; Y. Liu *et al.*, 2008; Rajput *et al.*, 2013).

Mycotoxin analysis (data not shown) indicated that mycotoxins were below the maximum levels set for aflatoxin B_1 in poultry feed (0.02 mg/kg) and the guidance values set for other mycotoxins by the European Union (European Commission, 2013, 2006, 2003, 2002).

Carotenoid analysis indicated that feed composition plays an important role in the final carotenoid content of the different tissues. The HC diet contained the highest levels of zeaxanthin, β-carotene and β-cryptoxanthin, whereas the commercial diet supplemented with color additives contained the highest levels of lutein. Similar proportions of these carotenoids were found in the chicken livers, with the exception of β-carotene, which was not detected in livers from any of the diet groups, reflecting its conversion into retinol for storage. The levels of β-cryptoxanthin, which also has PVA activity, remained high in livers from chickens fed on the HC diet. This suggests the preference of the enzyme β-carotene 15,15'-dioxygenase 1, for β-carotene as a substrate (Kim and Oh, 2010, 2009). Furthermore, β-cryptoxanthin levels in livers from chickens fed on the HC diet were higher than those originally present in the HC feed, suggesting that the polar nature of this molecule may facilitate more efficient transfer from the feed to the chicken tissues (Liu *et al.*, 2012). A high concentration of β-cryptoxanthin was detected in yolks from hens fed on a diet based on maize biofortified with β-cryptoxanthin (Liu *et al.*, 2012), and the β-cryptoxanthin concentration in yolks and chicken livers was higher than the initial β-cryptoxanthin content in the feed in hens fed on a biofortified orange-maize diet (Heying *et al.*, 2014). Even so, β-cryptoxanthin was not detected in livers from chickens fed on the control diet or was found at low levels in livers from chickens fed on the commercial diet (0.26 ± 0.05 μg/g fd). This probably reflects the conversion of β-cryptoxanthin into retinol because those diets contained less β-carotene than the HC feed. Alternatively, the difference in bioavailability may indicate a food matrix effect, in which carotenoids provided as intrinsic components are released more slowly and therefore absorbed over a longer duration for more efficient assimilation. Non-PVA carotenoids supplied to hens as biofortified maize are more efficiently assimilated than the same carotenoids provided as a commercial maize diet (Moreno *et al.*, 2016). Only lutein and zeaxanthin were detected in

the serum, which may reflect the preferential transport of these carotenoids in the circulation. Chicken serum contains more HDL than LDL, and β-carotene is mainly carried by LDL whereas lutein and zeaxanthin are carried by HDL (Heying *et al.*, 2014; Parker, 1996).

Chickens fed on the HC diet accumulated more retinol in the liver, but not in the serum. The liver is a major storage tissue for vitamin A whereas the serum is the mobile phase for carotenoids (Jlali *et al.*, 2012). Our results are consistent with a previous study showing that chickens fed on the HC diet had 1.72–fold more retinol in the liver than chickens fed on the control diet and 1.42–fold more than those fed on the commercial diet with color additives (Nogareda *et al.*, 2016). Here we found that chickens fed on the HC diet had 1.34–fold more retinol in the liver than chickens fed on the control diet and 1.28–fold more than those fed on the commercial diet with color additives. Despite the slightly smaller fold change, our absolute retinol levels were higher for all treatments compared to the previous study (Nogareda *et al.*, 2016), which may reflect the storage conditions of the feed which may lead to the loss of carotenoids over time (Jintasataporn and Yuangsoi, 2012).

Consumers usually reject or accept poultry meat based on its appearance, color being one of the major contributing components to this quality attribute. Skin color is crucial for the marketing of whole parts whereas meat color is important for the marketing of skinless cut up pieces (Fletcher, 2002). The chicken is the only poultry species in which pigmentation of products can be desired by consumers, although preferences vary by region and by culture (Grashorn, 2016). Some consumers can perceive the yellow color of chicken meat negatively because it is associated with an old bird or rancidity while others can consider it positively because it is associated with better health (Grashorn, 2016; Kennedy *et al.*, 2002). The intensity of the skin color was measured using the CIELab trichromatic system (CIE, 2004) to provide values for lightness (L*), redness (a*) and yellowness (b*). The redness and yellowness values were higher in chickens fed on carotenoid-enriched diets compared to control diet, and the values in the footpad were higher than those in the breast and thigh muscles after slaughter. Higher pigmentation levels in shank compared to breast were previously reported in experiments in which broilers were fed on diets supplemented with natural and synthetic pigments (Liu *et al.*, 2008). The color parameters vary according to the tissue, e.g. lower yellowness was detected when the target tissue was the fat vein (Castañeda *et al.*, 2005), and the production conditions

(e.g. higher yellowness was found when broilers were reared under intensive conditions) (Sirri *et al.*, 2010).

Previous experiments have shown that the levels of lutein were 2–fold higher in breast muscle from chickens fed on the HC diet compared to the control whereas there were no differences in fat, breast and thigh skin, and thigh muscle between the two diets. A more substantial difference was found in zeaxanthin levels, which were 7–fold higher in fat and thigh muscle, 11–fold higher in thigh skin, 20-fold higher in breast skin, and 22–fold higher in breast muscle from chickens fed on the HC diet compared to the control. β-Carotene levels were 11–fold higher in breast muscle from chickens fed on the HC diet compared to the control whereas it was not detected in fat, breast and thigh skin, and thigh muscle in any case. However, β-carotene epoxides were found in those tissues in chickens fed on the HC diet, suggesting that β-carotene was accumulated initially but it was metabolized into downstream derivatives. Finally, β-cryptoxanthin was only detected in breast muscle from chickens fed on the HC diet (Nogareda *et al.*, 2016).

Chickens fed on the commercial diet supplemented with color additives had higher breast and thigh yellowness compared to those fed on the HC diet, which can be explained by the presence of the lutein-rich marigold flower extract in this formulation (Breithaupt *et al.*, 2003). Lutein increases the yellowness of chicken meat, as reported for broilers fed on a diet supplemented with 200 mg/kg lutein (Rajput *et al.*, 2013). Yellowness is a good indicator of yellow xanthophyll content in the feed (Pérez-Vendrell *et al.*, 2001). Despite the better absorption of synthetic pigments (apo-ester and canthaxanthin), it seems that natural pigments (marigold flowers and red paprika extracts) are more efficiently distributed into tissues, because natural pigments increased skin yellowness more efficiently than synthetic pigments (Castañeda *et al.*, 2005).

A positive correlation between skin and raw breast meat yellowness was observed when Ross 508 broilers were reared under commercial conditions and fed on diets supplemented with xanthophylls (Bianchi *et al.*, 2007). A slight correlation was observed in the carotenoid-enriched diet groups under our experimental conditions, but higher yellowness was found in breast skin than in breast muscle in those groups. Yellowness in breast and thigh muscles was also correlated in experiments in which Ross 308 and Ross 508 broilers were reared under intensive conditions, suggesting that a single measurement in one tissue is adequate to assess color development (Sirri *et*

al., 2010). Despite there was a correlation between the yellowness values of breast and thigh muscles in our study, the correlation values were not as high as those reported by these authors.

The results obtained from the analysis of color parameters suggest that breast and thigh muscles are appropriate target tissues to measure the pigmentation of broilers intended to be processed into retail cuts. Taking into account yellowness and redness, the meat obtained from chickens reared on the HC diet was similar in appearance to the meat obtained from chickens reared on the commercial diet supplemented with marigold flowers and red paprika extracts. Thus, HC maize is a suitable feed component to achieve the golden skin color which is desired by the consumer in some markets, avoiding or reducing the need for supplementary pigments and thus reducing feed costs.

High-carotenoid maize had no adverse effects on poultry, and it resulted in similar growth and health parameters to its near isogenic wild-type line and the commercial maize supplemented with color additives, in addition to providing PVA carotenoids. Chickens fed on the HC diet developed similar pigmentation to those fed on the commercial diet supplemented with color additives, although the latter had greater yellowness values due to the high levels of lutein in the feed. We conclude that HC maize is a suitable alternative to color additives in the poultry production industry.

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CHAPTER III

Provitamin A carotenoids from an engineered high-carotenoid maize are bioavailable and zeaxanthin does not compromise β-carotene absorption in poultry

Joana Díaz-Gómez, Jose A. Moreno, Eduardo Angulo, Gerhard Sandmann, Changfu Zhu, Teresa Capell and Carmina Nogareda

Transgenic Research (2017),

DOI 10.1007/s11248-017-0029-y

Provitamin A carotenoids from an engineered high-carotenoid maize are bioavailable and zeaxanthin does not compromise β-carotene absorption in poultry

Joana Díaz-Gómez [a, b], Jose A. Moreno [b], Eduardo Angulo [b], Gerhard Sandmann [c] , Changfu Zhu [d], Teresa Capell [d] and Carmina Nogareda [b]

[a] Food Technology Department, University of Lleida–Agrotecnio Center; [b] Animal Science Department; [c] Biosynthesis Group, Department of Molecular Biosciences, J. W. Goethe University, Max von Laue Str. 9, D-60438 Frankfurt, Germany; [d] Plant Production and Forestry Science Department; [a, b, d] University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain

Abstract

High-carotenoid (HC) maize, a biofortified staple crop which accumulates β-carotene, β-cryptoxanthin, lutein and zeaxanthin, was used as a feed component in a chicken feeding trial to assess the bioavailability of provitamin A (PVA) carotenoids in the kernel matrix compared to the synthetic and natural color additives routinely used in the poultry industry. We found that the PVA carotenoids in HC maize were not metabolized in the same manner: β-carotene was preferentially converted into retinol in the intestine whereas β-cryptoxanthin accumulated in the liver. We also considered the effect of zeaxanthin on the absorption of PVA carotenoids because zeaxanthin is the major carotenoid component of HC maize. We found that chickens fed on diets with low levels of zeaxanthin accumulated higher levels of retinol in the liver, suggesting that zeaxanthin might interfere with the absorption of β-carotene, although this observation was not statistically significant. Our results show that HC maize provides bioavailable carotenoids, including PVA carotenoids, and is suitable for use as a feed component.

Keywords

Chicken, bioavailability, metabolic engineering, pigments, β-carotene

1. Introduction

Carotenoids are isoprenoids classified as carotenes (hydrocarbons) or xanthophylls (oxygenated derivatives) (Namitha and Negi, 2010). Some carotenoids possess provitamin A (PVA) activity (e.g. α-carotene, β-carotene and β-cryptoxanthin) whereas others act as antioxidants but cannot be converted into vitamin A (e.g. zeaxanthin and lutein) (Farré *et al.*, 2010). Humans acquire dietary carotenoids predominantly from plant-based foods. PVA carotenoids are important nutrients that are required to prevent vitamin A deficiency (VAD), whereas other carotenoids provide more general health-promoting antioxidant activity (Bramley, 2003; Institute of Medicine, 2001).

Staple crops with enhanced carotenoid levels have been developed through conventional breeding (e.g. potato, maize, cassava and pumpkin) or genetic engineering (e.g. rice, maize, potato and tomato) and these could help to maintain the health of populations at risk of vitamin A deficiency (VAD) without the need to fortify processed foods or provide supplements (Bai *et al.*, 2011; Gómez-Galera *et al.*, 2010; Saltzman *et al.*, 2013; Zhu *et al.*, 2013). High-carotenoid (HC) maize, which was generated through transformation of the South African white maize inbred line M37W, accumulates large amounts of β-carotene, β-cryptoxanthin, lutein and zeaxanthin (Zhu *et al.*, 2008).

In the human body, vitamin A is absorbed as preformed vitamin A from meat and dairy products or as PVA carotenoids from plants (Sanahuja *et al.*, 2013). Animal models are useful to evaluate carotenoid absorption and metabolism because target tissues such as the liver can be tested (Lee *et al.*, 1999). Chickens are a suitable model to study vitamin A absorption and availability because vitamin A and carotenoid metabolism is similar to humans, hence chickens are also susceptible to VAD (NRC, 1994; Pretorius and Schönfeldt, 2013). Like most other animals, chickens obtain carotenoids from their diet (Breithaupt, 2007; Moreno *et al.*, 2016). Carotenoids supplements are commonly used in poultry nutrition to achieve the skin pigmentation desired by many consumers and vitamin A is added as retinyl ester to ensure normal growth and development (Castañeda *et al.*, 2005; Yuan *et al.*, 2014).

Previous studies have established HC maize as an alternative to color additives and vitamin A fortificants in poultry feed formulations for laying hens (Moreno *et al.*, 2016) and broilers (Díaz-Gómez *et al.*, 2017a; Nogareda

et al., 2016). It is important to focus on nutrient bioavailability rather than on the total amount of nutrient provided, because the bioavailability (defined as the amount of an ingested nutrient that is available for utilization or storage) is influenced by dietary and physiological factors (Carbonell-Capella *et al.*, 2014; Sanahuja *et al.*, 2013). Furthermore, carotenoid absorption and metabolism are affected by interaction with other carotenoids (Furr and Clark, 1997; Yeum and Russell, 2002). For example, in chicks, the ingestion of more zeaxanthin inhibits the accumulation of lutein in plasma and non-retinal tissues (and vice versa) whereas the ingestion of more β-carotene inhibits the accumulation of zeaxanthin and lutein in plasma and most tissues (Wang *et al.*, 2010). The competition between lutein and β-carotene has been widely studied, but the role of zeaxanthin has not been investigated in detail (Mamatha and Baskaran, 2011; Yeum and Russell, 2002).

We therefore investigated the bioavailability of the PVA carotenoids β-carotene and β-cryptoxanthin supplied as intrinsic components of the HC maize compared to the same carotenoids provided by a near isogenic maize line supplemented with synthetic or natural color additives. We also considered whether the absorption of PVA carotenoids is affected by zeaxanthin, the most abundant carotenoid in HC maize.

2. Materials and methods

2.1. Diet preparation

The South African white maize inbred M37W and HC maize (Zhu *et al.*, 2008) were grown in an experimental field at the University of Lleida, Northeast Spain. After harvest, maize cobs were dried at 35 °C for 24 h in a drying chamber before milling (Ras® Mill, Romer® Labs Inc., Union, MO, USA). The diets were formulated and prepared at the Mas de Bover Research Center (IRTA, Reus, Spain) according to National Research Council recommendations (NRC, 1994). The feed composition was 58% maize, 18% soybean meal, 16% soybean hull and 4% soybean oil (Table 1). Vitamin A was included in the vitamin-mineral premix at a concentration of 8,000 IU/kg (2.4 mg retinol/kg) to ensure normal growth and development (Yuan *et al.*, 2014). The M37W control diet was prepared before the HC diet to avoid cross-contamination. Humidity, protein, fat, fiber and ash contents were measured following European Union (EU) regulations (European Commission, 2009). Mycotoxin levels were determined using enzyme immunoassay kits (Ridascreen®, R-Biopharm AG, Darmstadt, Germany).

Table 1. Diet composition.

Ingredient (g/kg)	Control diet	High-carotenoid diet
Control maize (M37W)	582.00	0
High-carotenoid maize	0	582.00
Soybean meal 47.5%	178.50	178.50
Soy hulls	158.50	158.50
Soybean oil	42.00	42.00
Monocalcium phosphate	12.28	12.28
Calcium carbonate	10.97	10.97
Sodium chloride	3.17	3.17
Sodium bicarbonate	1.19	1.19
Choline chloride 60%	0.42	0.42
L-Lysine chloride 79%	2.77	2.77
DL-Methionine 99%	3.33	3.33
L-Threonine 98%	0.47	0.47
Vitamin-mineral premix [1]	3.03	3.03
Vitamin premix grower [2]	1.04	1.04
Coccidiostat salinomycin 12%	0.52	0.52

Chemical analysis (g/kg)	Control diet	High-carotenoid diet
Crude protein	233.60	222.90
Crude fiber	40.80	26.70
Crude fat	112.40	111.30
Ash	43.30	47.40
Moisture	145.40	110.30

[1] Vitamin-mineral premix: vitamin D_3 1,700 IU/kg; vitamin B_1 2 mg/kg; vitamin B_2 6.4 mg/kg; vitamin B_6 3 mg/kg; vitamin B_{12} 0.02 mg/kg; vitamin E 50 mg/kg; vitamin K 3 mg/kg; folic acid 1 mg/kg; nicotinic acid 40 mg/kg; panthotenic acid 11.7 mg/kg; biotin 0.1 mg/kg; cooper 6 mg/kg; zinc 54 mg/kg; iron 40 mg/kg; manganese 77 mg/kg; selenium 0.45 mg/kg; iodine 2.28 mg/kg; BHT antioxidant 125 mg/kg.

[2] Vitamin premix grower: vitamin A 8,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

Four experimental diets were prepared: (a) M37W control diet supplemented with synthetic color additives (zeaxanthin, lutein and β-carotene); (b) M37W

control diet supplemented with synthetic color additives (lutein and β-carotene but not zeaxanthin); (c) M37W control diet supplemented with natural color additives (marigold flowers and red paprika extracts); (d) HC diet, based on high-carotenoid maize, with no color additives. The synthetic and natural color additives were introduced using a planetary mixer (Sammic, Azkoitia, Spain). Fortification was carried out in three batches of 4 kg to avoid carotenoid degradation, and each batch was used immediately after preparation.

The carotenoid content of the HC diet has been analyzed (Díaz-Gómez *et al.*, 2017a), and similar levels of PVA carotenoids as found in the HC diet were added to the control diets as synthetic (3.6 mg β-carotene/kg feed) or natural (1 g red xantophylls/kg feed and 0.3 g yellow xanthophylls/kg feed) color additives using a balance with a precision of 0.1 mg (Gram Precision, Barcelona, Spain). The purity of each synthetic compound and the marigold and red pepper extract compositions were taken into account in the calculations. We did not add β-cryptoxanthin to the feed formulation when synthetic additives were included, but its activity as a PVA carotenoid was taken into consideration in the calculations: 24 μg of β-cryptoxanthin is required to produce 1 μg retinol activity equivalent (RAE) (Institute of Medicine, 2001). The corresponding quantity was added as β-carotene (12 μg of β-carotene is required to produce 1 μg of RAE) (Institute of Medicine, 2001). The PVA carotenoid content of the diets is shown in Table 2.

Table 2. Provitamin A carotenoid composition (μg/g freeze-dried feed) of the diets. Values represent the mean and standard error (n = 3). Means within a column with no superscript in common are significantly different ($p < 0.05$). Zea, zeaxanthin; lut, lutein; β-car, β-carotene; β-cry, β-cryptoxanthin; nd, not detected.

	β-cry	β-car
High-carotenoid diet	1.04 ± 0.05 [a]	3.06 ± 0.17 [a]
Control diet + zea + lut + β-car	nd	2.72 ± 0.2 [ab]
Control diet + lut + β-car	nd	2.41 ± 0.11 [ab]
Control diet + natural additives	0.91 ± 0.05 [a]	2.24 ± 0.08 [b]

Different levels of zeaxanthin were introduced to determine whether zeaxanthin affects the absorption of PVA carotenoids. The zeaxanthin and lutein content of the diets is shown in Table 3. Yellow xanthophylls

(marigold flower extract, Capsantal EBS-40-NT) and red xanthophylls (red paprika extract, Capsantal FS-20-NT) were provided by Industrial Técnica Pecuaria S.A. (Barcelona, Spain) and synthetic additives (zeaxanthin, lutein and β-carotene) were provided by Cymit Química S.L. (Barcelona, Spain).

Table 3. Zeaxanthin and lutein levels in feed (μg/g freeze-dried), serum (μg/mL) and liver (μg/g freeze-dried) in chickens fed on four different diets. Values represent the mean and standard error (n = 3 for feed; n = 4 for serum and liver). Means within a row with no superscript in common are significantly different ($p < 0.05$). Zea, zeaxanthin; lut, lutein; β-car, β-carotene.

		High-carotenoid diet	Control diet + zea + lut + β-car	Control diet + lut + β-car	Control diet+ natural additives
lut	Feed	3.45 ± 0.12 ^{b}	1.45 ± 0.09 ^{c}	0.95 ± 0.05 ^{d}	5.76 ± 0.11 ^{a}
	Serum	1.09 ± 0.11 ^{b}	0.43 ± 0.02 ^{c}	0.33 ± 0.02 ^{c}	2.61 ± 0.17 ^{a}
	Liver	1.83 ± 0.47 ^{b}	0.38 ± 0.05 ^{c}	1.18 ± 0.29 ^{bc}	5.11 ± 0.31 ^{a}
zea	Feed	9.80 ± 0.58 ^{a}	3.46 ± 0.36 ^{b}	0.83 ± 0.04 ^{c}	2.01 ± 0.06 ^{bc}
	Serum	3.05 ± 0.32 ^{a}	0.92 ± 0.05 ^{b}	0.19 ± 0.00 ^{c}	0.72 ± 0.04 ^{bc}
	Liver	7.85 ± 1.77 ^{a}	1.62 ± 0.18 ^{b}	0.44 ± 0.10 ^{b}	0.56 ± 0.04 ^{b}

2.2. Experimental design and growth performance

Thirty-six 7-day-old male broiler chickens of the Ross 308 strain were obtained from a commercial hatchery, where they were fed on a wheat-barley-soy diet. The animals were weighed, wing-banded for identification and placed in a temperature-controlled room in the University of Lleida Animal Research Center. All chickens were fed on a basal diet with a low carotenoid content (based on M37W maize with no color additives) for the first 7 days to deplete and equalize the quantity of carotenoids in the animals. After this depletion period, four chickens were slaughtered to determine baseline serum and liver carotenoid concentrations. The remaining chickens (n = 32) were then randomly allocated to individual cages (40 cm width, 40 cm depth and 45 cm height, with a floor slope of 8%), eight cages per treatment. From day 14 until slaughter on day 28, the animals were fed on one of the four prepared diets described above. During the first week on the basal diet, feed and tap water were provided *ad libitum* through the same feeders and water dispensers, whereas individual feeders and water

dispensers were used for each experimental treatment during the remaining 2 weeks. No medication was administered during the feeding trial. Environmental conditions (temperature, humidity, lighting and ventilation) were monitored with a mini data logger (Testo, Lenzkirch, Germany) and changed according to the age of the birds, following National Research Council guidelines (NRC, 2011).

Body weights were measured each week of the experimental period and feed intake was measured every time the feeders were filled in accordance with standard industry methods, using a balance with a precision of 0.01 g (Kern, Balingen, Germany). After 2 weeks on the experimental diets, all the chickens were slaughtered on day 28 for gross necropsy. Pre-chilled whole liver, spleen and bursa of Fabricius were collected and weighed. Serum and freeze-dried (fd) liver samples were stored at –20 °C for carotenoid analysis.

The experimental procedure was approved by the Ethics Committee for Animal Experimentation of the University of Lleida and the Catalan Government (reference number DAAM 7692), following EU regulations (European Commission, 2007; European Parliament, 2010) as well as best practices recommended by the International Life Sciences Institute (ILSI, 2007).

2.3. Carotenoid analysis

The HC diet was analyzed before the feeding trial to calculate the quantities of PVA carotenoids to be added to the control diets. Because the synthetic and natural color additives were introduced into the control diets in three batches, each batch was randomly sampled, and the samples from each batch were combined to obtain an aggregate sample for carotenoid analysis. Total carotenoids were extracted from freeze-dried samples in 20 mL methanol containing 12% KOH at 65 °C for 1 h. Lipophilic compounds were partitioned into 30% diethyl ether in petroleum ether, the upper phase was collected and the solvent was evaporated under a stream of nitrogen gas at 37 °C. For HPLC separation, samples were re-dissolved in 100 µL methanol/dichloromethane (50: 50, *v*/*v*) and a 20-µL aliquot was injected immediately. Compounds were separated on a 15-cm Nucleosil C18 3-µm column with an acetonitrile, methanol and 2-propanol mobile phase (85: 15: 5, *v*/*v*/*v*) at 20 °C. Samples were monitored with a Kontron 440 photodiode array detector with online registration of the spectra, and were identified by comparison with authentic reference compounds (Sandmann, 2002).

2.4. Colorimetric analysis

A compact portable colorimeter CM-700d (Konica Minolta, Tokyo, Japan) was used to measure the color according to the CIELab trichromatic system based on lightness (L*), redness (a*) and yellowness (b*) (CIE, 2004). Coordinate L* represents lightness ranging from 0 (black) to 100 (white), a* indicates the red/green component and b* indicates the yellow/blue component. The range of both chromatic components is between −128 and 128 (Sharifzadeh *et al.*, 2014). The illuminant D65, a viewing angle of 10° and an 8-mm mask were used for all measurements. Color evolution during the trial was measured twice per week in the footpad, which was cleaned before analysis. After slaughter, the color was measured in pre-chilled footpad, breast skin and breast and thigh muscles.

2.5. Statistical analysis

Analysis of variance (ANOVA) statistical tests and Tukey's honest significant difference (HSD) test were used for comparison of means (JMP® Pro 12 SAS institute, 2015). Differences among means were regarded as significant at $p < 0.05$. A Grubbs' test was used to detect significant outliers ($p < 0.05$). Variables expressed in percentages were normalized using the arcsine of the square root of the probability.

3. Results

3.1. Compositional analysis

The diets were substantially equivalent except for carotenoid levels according to the standard compositional analysis (Table 1). Mycotoxin analysis detected only fumonisins (FBs) and zearalenone (ZEA), at 0.32 and 0.18 mg FBs/kg feed and 5.16 and 1.83 μg ZEA/kg feed, in the control and HC diets, respectively. The mycotoxin levels were therefore below the thresholds set by EU regulations (European Commission, 2002, 2003, 2006, 2013).

3.2. Growth performance

There were no significant differences ($p > 0.05$) in productivity parameters (Table 4) or in organ weight (Table 5) among chickens fed on the different diets.

Table 4. Broiler production parameters. Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a column with no superscript in common are significantly different ($p < 0.05$). FCR, feed conversion ratio; zea, zeaxanthin; lut, lutein; β-car, β-carotene.

	Body weight (g)	Feed intake (g)	FCR
High-carotenoid diet	1242 ± 43 [a]	1325 ± 30 [a]	1.52 ± 0.05 [a]
Control diet + zea + lut + β-car	1206 ± 18 [a]	1368 ± 40 [a]	1.64 ± 0.03 [a]
Control diet + lut + β-car	1210 ± 26 [a]	1313 ± 31 [a]	1.55 ± 0.02 [a]
Control diet + natural additives	1202 ± 48 [a]	1300 ± 45 [a]	1.55 ± 0.04 [a]

Table 5. Effects of diets on the relative organ weight of chickens (expressed as % relative to whole live animal weight). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a column with no superscript in common are significantly different ($p < 0.05$). Zea, zeaxanthin; lut, lutein; β-car, β-carotene.

	Liver	Spleen	Bursa of Fabricius
High-carotenoid diet	2.12 ± 0.10 [a]	0.09 ± 0.01 [a]	0.20 ± 0.01 [a]
Control diet + zea + lut + β-car	1.96 ± 0.06 [a]	0.08 ± 0.00 [a]	0.24 ± 0.01 [a]
Control diet + lut + β-car	1.92 ± 0.03 [a]	0.08 ± 0.01 [a]	0.25 ± 0.01 [a]
Control diet + natural additives	1.98 ± 0.08 [a]	0.08 ± 0.01 [a]	0.21 ± 0.02 [a]

3.3. Carotenoid and retinol levels

The β-carotene levels were quite similar in all the diets, although they were slightly lower in the control diet with natural additives (Table 1). Only the HC and control diet with natural additives contained β-cryptoxanthin, and there was no significant difference between the levels in each diet ($p > 0.05$). As expected, there were significant differences ($p < 0.05$) in zeaxanthin and lutein levels among the diets, given that different amounts of zeaxanthin and lutein were deliberately added to the control diet (Table 2). The HC diet contained the highest amount of zeaxanthin whereas the control diet with natural additives contained the highest amount of lutein. Violaxanthin was only detected in the HC diet (0.11 ± 0.01 μg/g fd feed).

The differences in zeaxanthin and lutein levels in the feed were also observed in the serum and liver samples (Table 2). The highest zeaxanthin levels were detected in the serum and liver of chickens fed on the HC diet, whereas the highest lutein levels were found in the serum and liver of chickens fed on the control diet with natural additives. Violaxanthin was not detected in the serum from chickens in any of the diet groups, but violaxanthin and/or similar polar epoxides were detected in the liver. Chickens fed on the HC diet had significantly higher levels of polar epoxides in the liver compared to the other diets (0.94 ± 0.13 μg/g fd liver; $p < 0.05$). The control diets with natural or synthetic additives contained similar levels of these compounds (0.32–0.50 μg/g fd liver). We detected β-cryptoxanthin only in the serum and liver from chickens fed on the HC diet (0.16 ± 0.02 μg/mL serum, 5.69 ± 1.21 μg/g fd liver). We did not detect β-carotene in the serum or liver of any chickens in any of the diet groups. However, β-carotene epoxides were detected in the liver samples from chickens fed on the HC diet (1.33 ± 0.39 μg/g fd liver). There were no significant differences in retinol levels (Fig. 1) in the serum or liver among chickens fed on the different diets ($p > 0.05$).

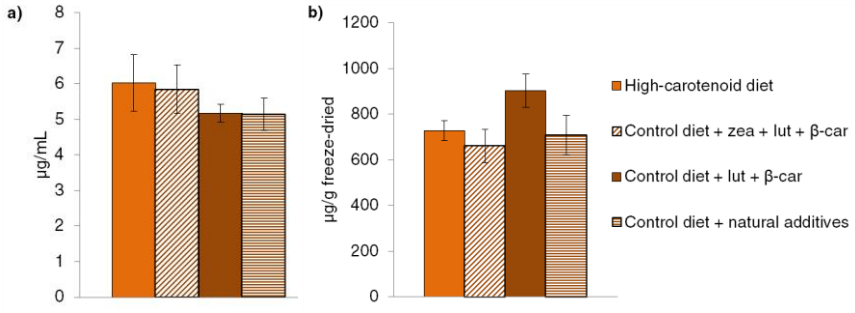

Figure 1. Retinol levels in **(a)** serum and **(b)** liver of chickens fed on four different diets. Values represent the mean and standard error (n = 4). Zea, zeaxanthin; lut, lutein; β-car, β-carotene.

Nevertheless, chickens fed on the control diet with synthetic additives lacking zeaxanthin had the highest retinol levels in the liver. Principal component analysis (PCA) revealed a trend towards higher retinol levels in liver when PVA carotenoids were supplied by diets containing low levels of zeaxanthin (Fig. 2).

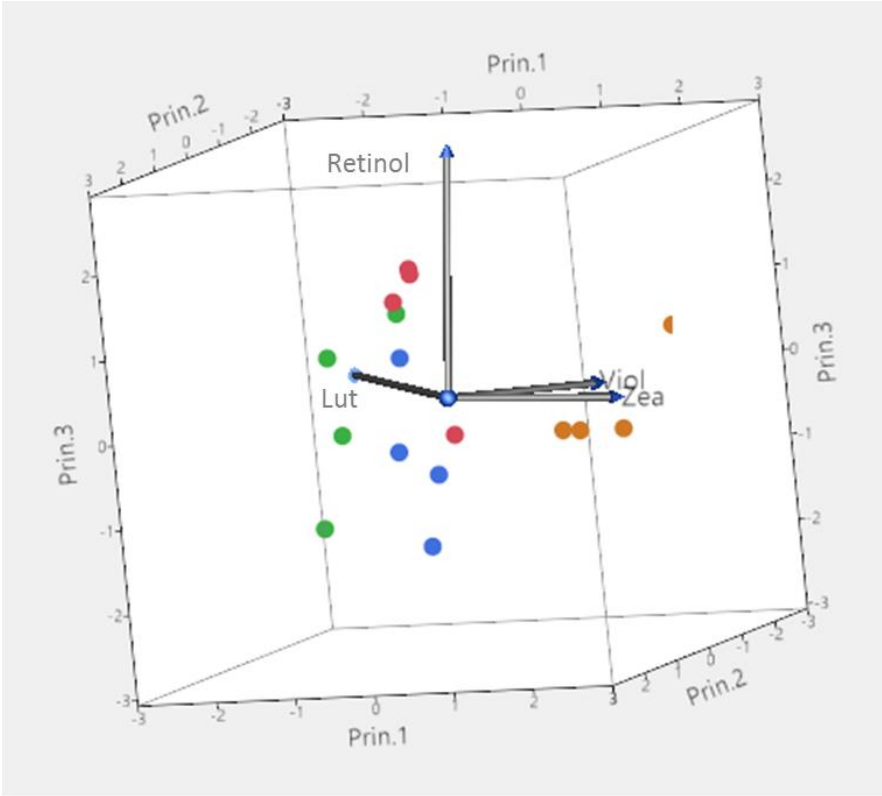

Figure 2. Principal component analysis of carotenoid and retinol levels in the livers of chickens fed on four different diets. Zea, zeaxanthin; lut, lutein; β-car, β-carotene; viol, violaxanthin.

3.4. Color parameters

Significant differences ($p < 0.001$) in redness and yellowness among chickens fed on the different diets were observed 3 days into the feeding trial (day 17), whereas significant differences ($p < 0.001$) in lightness were not found until a week (day 21) (Fig. 3). The lowest redness and yellowness were found in chickens fed on the control diet with synthetic additives lacking zeaxanthin.

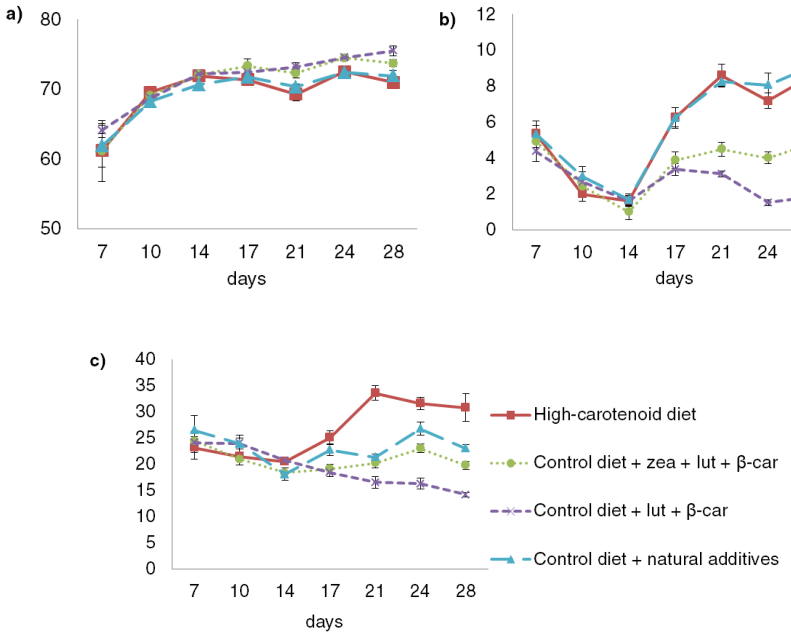

Figure 3. Color evolution in the footpad of chickens fed on the different diets for 2 weeks (14–28 days), using the CIELab trichromatic system to score **(a)** lightness (L*), **(b)** redness (a*) and **(c)** yellowness (b*). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Zea, zeaxanthin; lut, lutein; β-car, β-carotene.

The differences in color parameters found during the trial were corroborated when color was measured after slaughter in pre-chilled footpad, breast skin, breast muscle and thigh muscle (Table 6). There were no significant differences in lightness ($p > 0.05$), except for footpad lightness ($p < 0.001$), with the highest values found in chickens fed on the control diet with synthetic additives lacking zeaxanthin. There were significant differences in redness and yellowness in breast skin and in breast and thigh muscles ($p < 0.05$). Chickens fed on the HC diet had the highest yellowness score whereas chickens fed on the control diet with natural additives had the highest redness score. This difference was more evident in the breast skin and breast muscle yellowness score. The lowest redness and yellowness scores in breast and thigh muscles were observed in chickens fed on the control diet with synthetic additives lacking zeaxanthin.

Table 6. Color determination in footpad, breast skin, breast muscle and thigh muscle of chickens fed on the different diets after slaughter, using the CIELab trichromatic system to score lightness (L*), redness (a*) and yellowness (b*). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a row with no superscript in common are significantly different ($p < 0.05$). Zea, zeaxanthin; lut, lutein; β-car, β-carotene.

		High-carotenoid diet	Control diet + zea + lut + β-car	Control diet + lut + β-car	Control diet + natural additives
Footpad	L*	70.98 ± 0.65 ^{c}	73.74 ± 0.49 ^{ab}	75.54 ± 0.72 ^{a}	71.91 ± 0.84 ^{bc}
	a*	8.68 ± 0.43 ^{a}	4.87 ± 0.43 ^{b}	1.85 ± 0.29 ^{c}	9.17 ± 0.57 ^{a}
	b*	30.86 ± 2.64 ^{a}	19.87 ± 0.80 ^{bc}	14.23 ± 0.42 ^{c}	23.09 ± 0.71 ^{b}
Breast skin	L*	66.20 ± 1.60 ^{a}	66.24 ± 1.00 ^{a}	68.92 ± 1.16 ^{a}	65.19 ± 0.68 ^{a}
	a*	2.50 ± 0.45 ^{a}	1.24 ± 0.29 ^{a}	1.28 ± 0.65 ^{a}	3.47 ± 0.77 ^{a}
	b*	12.99 ± 1.55 ^{a}	6.05 ± 0.89 ^{b}	3.97 ± 1.18 ^{b}	7.04 ± 0.74 ^{b}
Breast muscle	L*	48.41 ± 0.89 ^{a}	48.91 ± 0.73 ^{a}	49.43 ± 1.00 ^{a}	48.12 ± 0.81 ^{a}
	a*	2.45 ± 0.25 ^{a}	1.00 ± 0.15 ^{b}	0.47 ± 0.22 ^{b}	2.91 ± 0.28 ^{a}
	b*	7.42 ± 0.41 ^{a}	3.91 ± 0.54 ^{bc}	2.74 ± 0.39 ^{c}	5.38 ± 0.63 ^{b}
Thigh muscle	L*	51.99 ± 0.71 ^{a}	51.40 ± 0.37 ^{a}	53.29 ± 0.69 ^{a}	51.67 ± 0.82 ^{a}
	a*	4.76 ± 0.47 ^{ab}	3.49 ± 0.17 ^{bc}	2.19 ± 0.34 ^{c}	5.50 ± 0.29 ^{a}
	b*	6.80 ± 1.02 ^{a}	4.18 ± 0.81 ^{ab}	2.26 ± 0.78 ^{b}	5.14 ± 1.35 ^{ab}

4. Discussion

The xanthophylls lutein and zeaxanthin are found in yellow maize, whereas PVA carotenoids such as β-carotene are not present at significant levels (Davis *et al.*, 2008). Novel crops with enhanced carotenoid levels, such as HC maize, offer an alternative to the fortification of processed food products or the provision of feed additives (Gómez-Galera *et al.*, 2010; Nogareda *et al.*, 2016). Nevertheless, carotenoid bioavailability in novel crops must be assessed because the efficiency of nutrient uptake from foods is influenced by many factors, including the food matrix, food processing and gastrointestinal absorption (Sanahuja *et al.*, 2013). We therefore tested the bioavailability of the PVA carotenoids in HC maize compared to the same carotenoids provided by its near isogenic line (M37W) (Zhu *et al.*, 2008) supplemented with synthetic or natural color additives. We also investigated

whether zeaxanthin, a major carotenoid in HC maize, affects the absorption of PVA carotenoids.

HC maize as a feed component did not adversely affect any of the criteria we evaluated (e.g. production parameters and organ weight), which confirms the results of earlier poultry nutrition experiments with this variety (Díaz-Gómez *et al.*, 2017a; Moreno *et al.*, 2016; Nogareda *et al.*, 2016). The performance of the control diet supplemented with synthetic and natural color additives also agree with earlier trials, confirming that the additives did not affect body weight or feed consumption (Liu *et al.*, 2008; Pérez-Vendrell *et al.*, 2001). We added synthetic β-carotene to the M37W control diets to achieve the same levels present in the HC diet, but there was some loss during feed preparation and storage despite the preparation of diets in three batches to avoid carotenoid degradation (Jintasataporn and Yuangsoi, 2012). Similar losses of vitamin A have been reported in supplemented poultry diets, although the losses were not statistically significant (Pretorius and Schönfeldt, 2013). As previously reported for other biofortified crops (e.g. maize and pumpkin), the carotenoids in HC maize are likely to be protected from degradation by interactions with the food matrix (Díaz-Gómez *et al.*, 2017b).

Zeaxanthin and lutein are the major carotenoids in HC maize and marigold flowers, respectively, and this explains why those carotenoids were present at the highest levels in the HC diet and the control diet with natural additives, respectively (Breithaupt, 2007; Naqvi *et al.*, 2009). The small amount of zeaxanthin found in the control diet supplemented with synthetic carotenoids lacking zeaxanthin came from the M37W maize, which contains traces of carotenoids, primarily lutein and zeaxanthin (Naqvi *et al.*, 2009). As expected, xanthophyll levels in the serum and liver were primarily determined by feed composition: chickens fed on the HC diet and control diet with natural additives accumulated the highest serum/liver zeaxanthin and lutein levels, respectively. Violaxanthin and/or similar polar epoxides were found in livers from chickens fed on all four diets despite only being detectable in the HC diet formulation, which may reflect the metabolic conversion of zeaxanthin to violaxanthin. Nutrients integrated in the food matrix are released more slowly and absorbed over a longer period for more efficient assimilation (Moreno *et al.*, 2016), and for that reason violaxanthin levels in livers from chickens fed on the HC diet were 8.5–fold more concentrated than the levels in the HC diet itself. This efficient transfer was

previously observed in eggs laid by hens fed on a diet based on BKT maize, a biofortified maize rich in ketocarotenoids (Moreno *et al.*, 2016).

The PVA carotenoids in HC maize (β-carotene and β-cryptoxanthin) were bioavailable and contributed to the storage of vitamin A in the liver as retinol (Moreno *et al.*, 2016). Consequently, β-carotene was not detected in the serum or liver samples from any of the diet groups because it was converted efficiently into retinol. However, in chickens fed on the HC diet, β-carotene epoxides were detected in liver, and these could have a protective role due to their antioxidant activity (Gurak *et al.*, 2014). In contrast, β-cryptoxanthin was detected in liver samples but only from chickens fed on the HC diet, and the concentration of this PVA carotenoid was higher than originally present in the feed. The higher β-cryptoxanthin levels in the liver may reflect the fact that β-carotene is a better substrate for β-carotene 15,15'-dioxygenase (BCDO1) (Kim and Oh, 2009, 2010). These results support our earlier study in chickens in which the food matrix was shown to play an important role in the bioavailability of carotenoids, given that β-cryptoxanthin in HC maize is provided as an intrinsic component (Díaz-Gómez *et al.*, 2017a). Regarding vitamin A absorption, it would have been ideal to study β-carotene and β-cryptoxanthin conversion to retinol separately. However, this is not practical as PVA carotenoids in HC maize are provided within the kernel matrix in which zeaxanthin and lutein are also present.

The efficiency of β-carotene transfer from the feed to liver was highest in the control diet with synthetic additives lacking zeaxanthin, followed by the control diet with natural additives, the control diet with synthetic additives including zeaxanthin, and finally the HC diet. Zeaxanthin appears to attenuate the bioaccessibility of β-carotene, and the underlying mechanism may be competition for solubilization in mixed micelles and absorption in the intestine (Furr and Clark, 1997; Yeum and Russell, 2002). Bioaccessibility is the amount of an ingested nutrient that is released from the food matrix in the gastrointestinal tract and becomes available for absorption, whereas bioavailability is the amount of an ingested nutrient that is available for utilization or for storage (Carbonell-Capella *et al.*, 2014; Díaz-Gómez *et al.*, 2017b). Carotenoids can interact with each other during absorption, metabolism and transport (Bohn, 2008; Yeum and Russell, 2002). Xanthophylls such as zeaxanthin and lutein are absorbed more efficiently than β-carotene in chickens and humans because they are more polar (Furr and Clark, 1997; Na *et al.*, 2004). The location of carotenoids in the lipid droplet also affects its absorption because oxygenated carotenoids

(xanthophylls) are located near the surface whereas hydrocarbon carotenoids (carotenes) are located within the core (Borel *et al.*, 1996; Yeum and Russell, 2002). However, the bioaccessibility of PVA carotenoids is minimally affected by maize xanthophylls, but their uptake by cells is inhibited by lutein in a dose-dependent manner (Thakkar and Failla, 2008). Different concentrations of maize xanthophylls did not have any effect on the bioefficacy (retinol/β-carotene total) (Tanumihardjo, 2002) of PVA carotenoids in Mongolian gerbils (Davis *et al.*, 2008), but these animals are not a good model for xanthophyll absorption because they only absorb and store small quantities of these molecules (Escaron and Tanumihardjo, 2006; Molldrem and Tanumihardjo, 2004). Carotenoids may compete for specific transporters (e.g. class B type I scavenger receptors) at the level of individual enterocytes (Bohn, 2008; Reboul, 2013).

Interactions among carotenoids in mammals are also observed when PVA carotenoids are cleaved by an oxygenase to produce retinal (Lietz *et al.*, 2010). The enzyme BCDO1 preferentially cleaves PVA carotenoids whereas β-carotene-9',10'-dioxygenase 2 (BCDO2) also metabolizes non-PVA carotenoids such as zeaxanthin (Palczewski *et al.*, 2014). Zeaxanthin can interact with the BCDO1, as shown by the limited conversion of intestinal β-carotene to vitamin A in vitro (Grolier *et al.*, 1997). Nevertheless, the supplementation of poultry diets with xanthophylls (20 or 40 mg/kg) did not have any effect on *BCDO1* mRNA expression in the liver or small intestine (Gao *et al.*, 2016). Therefore, zeaxanthin appears to interact with β-carotene at the levels of solubilization and absorption rather than competing for enzymatic cleavage.

We found that higher lutein levels were present in the control diet with natural additives compared to the other diets, but the HC diet resulted in tissues with higher yellowness scores. This may reflect the high zeaxanthin content of the HC diet. Higher levels of yellow rather than red xanthophylls are routinely used in the poultry industry, but in our trial we used more red than yellow xanthophylls to achieve PVA carotenoid levels similar to those present in the HC maize. The higher yellowness scores in chickens fed on the HC diet show that xanthophylls from HC maize can achieve the golden skin color desired by many consumers, so HC maize could replace xanthophylls commonly used in the poultry industry. Redness was slightly higher in chickens fed on the control diet with natural additives than in chickens fed on the HC diet due to the higher proportion of red

xanthophylls we used to achieve PVA carotenoid levels similar to those present in the HC diet.

We established that PVA carotenoids supplied as intrinsic components of HC maize are bioavailable at least to the same extent as synthetic compounds and natural extracts, but the metabolism was not the same for all types of molecules. Specifically, β-carotene was preferentially converted into retinol in the intestine whereas β-cryptoxanthin was accumulated in the liver. Lutein, zeaxanthin and β-carotene were bioavailable to the extent that was predicted based on their levels in the HC feed, whereas β-cryptoxanthin and violaxanthin were found at higher levels than originally present in the feed, highlighting the need to evaluate individual nutrient bioavailability rather than the total amount of nutrient provided. Despite the high zeaxanthin content in the HC diet, which interferes with the absorption of β-carotene, the retinol levels in chicken liver remained high. Furthermore, chickens fed on the HC diet had higher yellowness scores for breast and thigh muscles. We therefore conclude that HC maize provides bioavailable carotenoids, including PVA carotenoids, and is suitable for development as a feed component in the poultry industry.

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CHAPTER IV

Quality and sensory shelf life of raw chicken meat obtained from broilers fed on a diet based on carotenoid-biofortified maize

Joana Díaz-Gómez, Maria-Paz Romero, Carmina Nogareda, Antonio J. Ramos and Robert Soliva Fortuny

Submitted to: *Journal of Food Science*

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Joana Díaz-Gómez [a, b], Maria-Paz Romero [a], Carmina Nogareda [b], Antonio J. Ramos [a] and Robert Soliva-Fortuny [a]

[a] Food Technology Department, University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain;
[b] Animal Science Department, University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain

Abstract

Synthetic and natural color pigments are routinely used as feed components in the poultry industry to achieve desired skin pigmentation. High carotenoid (HC) maize, which was engineered to accumulate β-carotene, lutein and zeaxanthin, is a cost-effective alternative to feed supplementation. The objective of the current study was to evaluate the shelf life of raw chicken meat through a sensory analysis and subsequent survival analysis. Meat samples were obtained from chickens fed on three different diets: high-carotenoid (based on HC maize), control (based on its near-isogenic line) and commercial (based on standard yellow maize plus color additives). Breast and leg quarters (which were previously frozen, thawed and stored under refrigerated conditions) were olfactory tested after between 1 and 15 days of storage by a consumer panel. Microbial counts (*Pseudomonas* spp. and total aerobic mesophilic bacteria) and physical quality parameters (pH, color and firmness) were evaluated. Considering 25% of consumers rejecting the product, shelf lives were estimated at between 4.5 and 10 days. Breast and leg quarters from chickens fed on the HC diet were equally accepted by the panel than samples from the other two diets until day 9 ($p > 0.05$). Spoilage bacteria increased progressively from 1 to 9 days (up to 10^6–10^7 cfu/g). Meat from chickens fed on the carotenoid-enhanced diets exhibited more intense red and yellow color ($p < 0.05$), while firmness was similar among treatments. Considering the commercial chicken as a standard for meat quality and shelf life, we conclude that meat from chickens fed on a biofortified maize diet has a good quality and shelf life.

Keywords

Poultry meat, pigments, metabolic engineering, sensory analysis, survival analysis

1. Introduction

Consumer acceptance of poultry meat and its products is mainly related to appearance, as color and glossiness are associated with quality parameters such as freshness, flavor and texture (Totosaus *et al.*, 2007). Skin color mainly depends on dietary carotenoids present in the feed composition (Pérez-Vendrell *et al.*, 2001; Sirri *et al.,* 2010). Like most other animals, chickens cannot synthesize carotenoids. Thus, feed is supplemented with synthetic or natural pigments, which are deposited in the skin and subcutaneous fat, conferring the characteristic golden pigmentation (Breithaupt, 2007; Pérez-Vendrell *et al.*, 2001). More than 21 billion chickens were produced globally in 2014, China being the first producer, followed by the United States (FAOSTAT, 2015). In those markets a golden skin color is preferred by many consumers, as it is often associated with a healthy chicken (Tarique *et al.*, 2013; Williams, 1992). Therefore, it makes the addition of color pigments to poultry feed necessary, which increases production costs.

Novel crops with enhanced carotenoid content, such as high-carotenoid (HC) maize, which accumulates β-carotene, lutein and zeaxanthin (Zhu *et al.*, 2008), serve as an alternative strategy to feed supplementation. HC maize has previously been shown to provide bioavailable carotenoids and to confer a similar skin pigmentation to color additives commonly used in the poultry industry (Díaz-Gómez *et al.*, 2017; Nogareda *et al.*, 2016). However, the effect of HC maize-based feed on chicken meat quality and shelf life is unknown.

Susceptibility of chicken meat to spoilage, commonly detected by sensory and microbiological analyses, represents a potential health risk (Dainty, 1996). Many food products may remain microbiologically safe to eat, but changes in their sensory characteristics could cause consumer rejection (Calle *et al.*, 2006; Hough *et al.*, 2003). Consequently, sensory evaluation is an important factor in the determination of shelf life in food products. Survival analysis methods are applied to shelf life of foods to evaluate the probability of a consumer accepting a product after a certain storage time (Kleinbaum, 1996). Thus, they focus on consumer rejection rather than on product deterioration (Calle *et al.*, 2006; Curia *et al.*, 2005; Hough *et al.*, 2003). Several

shelf life studies based on survival analysis have been carried out, but chicken meat is not usually studied. Sensory analysis can be influenced by the conditions under which samples are obtained and stored. Freezing, commonly used in the meat industry to preserve meat quality and safety over longer periods, can affect meat quality due to changes in biochemical and physical parameters (Leygonie *et al.*, 2012; Zhuang and Savage, 2013). Consequently, it is necessary to evaluate whether freezing, frozen storage and subsequent thawing have some effect on quality measurements.

Therefore, the aim of this research was to estimate the sensory shelf life of breast and leg quarter meat obtained from chickens fed on the HC diet (based on HC maize) compared to the shelf life of chicken meat obtained from animals fed on a control diet (based on M37W maize) and a commercial diet (based on standard commercial yellow maize supplemented with natural color additives) through survival analysis methodology. Complementarily, total aerobic mesophilic bacteria and *Pseudomonas* spp. were measured as indicators of microbiological shelf life, and physical parameters (pH, color and texture) were determined to evaluate the effect of the diets on the chicken meat quality for both refrigerated and thawed samples. The chicken meat quality is the term used to describe the attributes of chicken meat that affect the decision of purchase. Our study includes the evaluation of appearance, smell and texture.

2. Materials and methods

2.1. Animals and dietary treatments

HC maize and its near isogenic line, the South African white maize inbred M37W, were grown in an experimental field in Northeastern Spain (Lleida, Catalonia) for two consecutive harvest seasons. Therefore, two animal feeding trials were carried out to have experimental replicates as the number of animals was limited by the quantity of maize harvested each year. As a result, the number of meat samples was also restricted.

The animals, 1-day-old male broiler chickens of the Ross 308 strain, were housed in four pens per treatment (24 broilers per treatment). They were fed on three diets including different types of maize from 0 to 35 days: control diet (based on M37W maize), HC diet (based on HC maize) and commercial diet (based on standard commercial yellow maize supplemented with natural color additives - marigold flowers and red paprika extracts -) (Díaz-Gómez *et*

al., 2017). The diets were provided as starter feed (52% maize, 36% soybean meal and 7% soybean oil) on days 0–8 and grower feed (58% maize, 18% soybean meal, 16% soybean hull and 4% soybean oil) on days 9–35 (Table 1). The diet formulations as well as environmental conditions were in accordance with the National Research Council recommendations (NRC 1994, 2011). The final body weight for chickens fed on the control, HC and commercial diets was 1831 ± 69, 1837 ± 56 and 1797 ± 57 g, respectively (Díaz-Gómez *et al.*, 2017). At the end of the trials (at 35 days), chickens were slaughtered in the facilities of the University of Lleida (Catalonia, Spain). Chickens were stunned with carbon dioxide prior to killing and bleeding. After slaughter, chickens were placed in a cold room at +12 °C where they were manually eviscerated, removing all the organs and weighing the liver, spleen and bursa of Fabricius. After evisceration, chicken carcasses were placed in a cold room at +4 °C where breast and leg quarter meat was removed, introduced in polyethylene self-sealing bags (García de Pou, Spain) separately and stored at +4 °C or -20 °C until needed for processing. Breast and leg quarter meat obtained was used to assess microbiological, sensory and physical parameters. Refrigerated samples were stored during 15 days and taken out every day (hereinafter referred to as refrigerated samples) whereas frozen samples were taken out at different times, thawed, deboned (if necessary) and stored under refrigerated conditions (hereinafter referred to as thawed samples) to achieve the desired storage period (Table 2). All analyses were performed within 1-2 months. All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida and the Catalan Government (reference numbers DAAM 7672 and DAAM 7743).

Table 1. Composition of starter (days 0–9) and grower (days 10–35) diets and chemical analysis of the grower diets (Díaz-Gómez *et al.*, 2017). HC, high-carotenoid.

Ingredient (g/kg)	Starter control	Starter HC	Starter commercial	Grower control	Grower HC	Grower commercial
Control maize	522.00	0	0	582.00	0	0
High-carotenoid maize	0	522.00	0	0	582.00	0
Commercial maize	0	0	522.00	0	0	582.00
Soybean meal 47.5%	361.00	361.00	361.00	178.50	178.50	178.50
Soy hulls	0	0	0	158.50	158.50	158.50
Soybean oil	68.00	68.00	68.00	42.00	42.00	42.00
Monocalcium phosphate	15.23	15.23	15.23	12.28	12.28	12.28
Calcium carbonate	14.55	14.55	14.55	10.97	10.97	10.97
Sodium chloride	3.47	3.47	3.47	3.17	3.17	3.17
Sodium bicarbonate	2.33	2.33	2.33	1.19	1.19	1.19
Choline chloride 60%	0.51	0.51	0.51	0.42	0.42	0.42
L-Lysine chloride 79%	2.69	2.69	2.69	2.77	2.77	2.77
DL-Methionine 99%	3.26	3.26	3.26	3.33	3.33	3.33
L-Threonine 98%	0.42	0.42	0.42	0.47	0.47	0.47
Vitamin-mineral premix [1]	3.03	3.03	3.03	3.03	3.03	3.03
Vitamin premix starter [2]	3.03	3.03	3.03	0	0	0
Vitamin premix grower [3]	0	0	0	1.04	1.04	1.04
Coccidiostat (cygro)	0.51	0.51	0.51	0	0	0
Coccidiostat salinomycin 12%	0	0	0	0.52	0.52	0.52
Mixture of color additives	0	0	0	0	0	0.93

Chemical analysis (g/kg)	Starter control	Starter HC	Starter commercial	Grower control	Grower HC	Grower commercial
Crude protein	-	-	-	218.50	219.60	190.50
Crude fiber	-	-	-	45.30	40.80	41.00
Crude fat	-	-	-	99.20	99.40	91.70
Ash	-	-	-	48.10	57.40	54.60
Moisture	-	-	-	143.90	102.90	124.00

[1] Vitamin-mineral premix: vitamin D_3 1,700 IU/kg; vitamin B_1 2 mg/kg; vitamin B_2 6.4 mg/kg; vitamin B_6 3 mg/kg; vitamin B_{12} 0.02 mg/kg; vitamin E 50 mg/kg; vitamin K 3 mg/kg; folic acid 1 mg/kg; nicotinic acid 40 mg/kg; panthotenic acid 11.7 mg/kg; biotin 0.1 mg/kg; cooper 6 mg/kg; zinc 54 mg/kg; iron 40 mg/kg;

manganese 77 mg/kg; selenium 0.45 mg/kg; iodine 2.28 mg/kg; BHT antioxidant 125 mg/kg.

[2] Vitamin premix starter: vitamin A 10,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

[3] Vitamin premix grower: vitamin A 8,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

Table 2. Storage time of chicken meat samples evaluated by the consumer panel in the first and second trials.

	Days of storage						
1st trial	1	4	6	7	8	9	11
2nd trial	1	4	7	9	11	13	15

2.2. Sensory shelf life analyses

2.2.1. Descriptive panel

The descriptive panel consisted of six members (50% female and 50% male, ages ranging between 45 and 65) with previous experience in sensory evaluation of chicken meat. Panelists were trained for 20 h in evaluating attributes related to appearance and aroma of breast and leg quarter meat obtained from retail market. These chicken cuts, which were stored at different times (from 0 to 10 days), were presented to the panelists as raw chicken meat. The descriptive analysis was conducted on two replications and the panelists evaluated the attributes related to appearance and aroma, quantifying on a rating scale from 0 to 10.

After the first animal feeding trial, breast and leg quarter meat samples were stored under refrigerated conditions and evaluated daily for 15 days. Every day, raw meat samples obtained from chickens fed on the different diets were presented to each panelist, six samples for panelist, corresponding to two different chicken cuts (breast and thigh meat) and three different treatments (control, HC and commercial diets). They evaluated overall appearance and smell attributes, quantifying on a rating scale from 0 to 10. Samples quantified lower than 5 were considered as rejected. These results were used to design the sensory test for the consumer panel.

2.2.2. Consumer panel

The sensory shelf life study was performed using a reversed storage design which consists of having all samples, each with a different storage time, available on the same day. Therefore, panelists were recruited on a single day to evaluate all samples (Hough, 2010). Samples previously thawed and deboned (if necessary) were prepared under refrigerated and hygienic conditions before each session and presented in black polypropylene portion containers closed with a lid (DART container corporation, Mason, MI, USA). A complete block design was used, randomizing both samples (which were coded with three-digit numbers) and order of presentation (Meilgaard *et al.*, 2007). All sensory evaluations were conducted in a standardized sensory testing room with individual booths under controlled conditions of light (6500 K daylight), temperature (20 °C) and relative humidity (70%).

Consumers were recruited among students and workers from the School of Agrifood and Forestry Science and Engineering (ETSEA, University of Lleida, Spain). Consumers were 67% female and 33% male, ages ranging between 18 and 65. As two animal feeding trials were performed, two sensory shelf life studies were carried out, with two tasting sessions in each trial, one to evaluate breast meat and the other to evaluate leg quarter meat. In the first trial there were 69 and 45 panelists for breast and leg quarter meat sensory analyses, respectively, while in the second trial there were 58 and 65 panelists for breast and leg quarter meat sensory analyses, respectively. Each panelist evaluated the odor of 7 raw samples with different storage times (Table 2), answering *yes* or *no* to whether they would normally consider the samples suitable for cooking, waiting a minute between sample and sample (Fig. 1). After performing the first trial, chicken meat did not show many perceptible changes, and therefore it was decided to extend the storage time of the samples in the second trial.

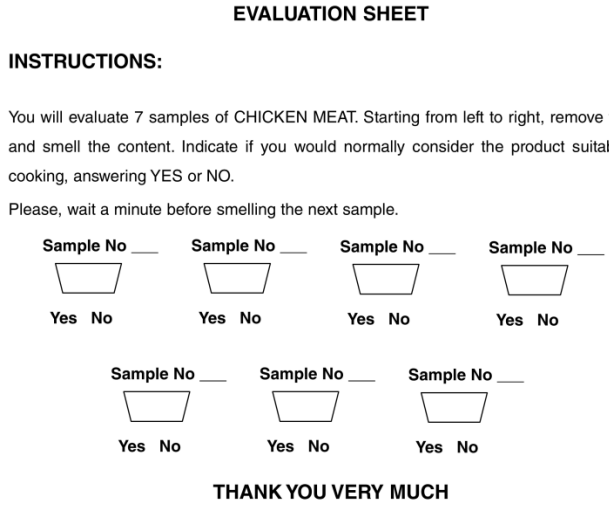


Figure 1. Evaluation sheet.

2.2.3. Survival analysis

Results obtained from the sensory analysis, in which consumers were asked if they would normally consider the samples suitable for cooking, were analyzed using the survival analysis methodology as described by Hough (2010). This methodology estimates the rejection function, which calculates the experimental percent rejection corresponding to each storage time. The best function was selected comparing the loglikelihood values of the log-normal, logistic and Weibull models (Hough, 2010). Log-normal distribution was selected to model rejection times for the data obtained from sensory analysis due to the good fitting performance.

2.3. Microbiological analyses

Microbiological analyses of breast and thigh meat were carried out at 1, 4, 7 and 9 days, in refrigerated and thawed samples. A sample of chicken meat (10 g) was transferred to a sterile stomacher bag and homogenized with 90 mL of buffered peptone water (Biokar diagnostics, Beauvais, France) for 60 s in a stomacher blender (IUL Instruments, Barcelona, Spain). Appropriate 10–fold serial dilutions were used for enumeration of total aerobic mesophilic bacteria on Plate Count Agar (Biokar diagnostics) and the plates were incubated at 30 °C for 72 h, according to UNE-EN ISO 4833-1 (ISO, 2014). The same procedure was used to determine *Pseudomonas* spp. on

Pseudomonas Agar Base (Oxoid, Hampshire, UK) with CFC selective supplement (Biokar diagnostics) and the plates were incubated at 25 °C for 48 h, according to UNE-EN ISO 13720 (ISO, 2011). The presumptive colonies of *Pseudomonas* spp. were then isolated in Nutritive Agar (Biokar diagnostics), incubated at 25 °C for 24 h, and confirmed by detection of cytochrome oxidase using Bactident® Oxidase strips (Merck Millipore, Madrid, Spain).

2.4. pH measurement

The pH determination in breast and thigh meat was performed at 1, 4, 7 and 9 days, in refrigerated and thawed samples, using a Crison pH-meter combined with an electrode 52/32 (Hach Lange Spain, Barcelona, Spain).

2.5. Color measurement

After slaughter, the color was measured in breast and thigh muscles in all animals, while it was only measured in two samples (breast and thigh meat) from each diet treatment during storage. The color determination was carried out at 1, 4, 7, 9 and 11 days, in refrigerated and thawed samples, using a compact portable colorimeter CM-700d (Konica Minolta, Tokyo, Japan) according to the CIELab trichromatic system as lightness (L*), redness (a*) and yellowness (b*) (CIE, 2004). Coordinate L* represents lightness ranging from 0 (black) to 100 (white), a* indicates the red/green component and b* indicates the yellow/blue component. The range of both chromatic components is between −128 and 128 (Sharifzadeh *et al.*, 2014). An illuminant D65, a 10° viewing angle and an 8-mm-diameter mask were used.

2.6. Texture measurement

The hardness was evaluated in breast meat through the Warner–Braztler (WB) shear test using a texture analyzer (TA-XT®, Stable Micro Systems Ltd., England, UK). This parameter was evaluated at 1, 4, 7, 9 and 11 days, in refrigerated and thawed samples. For texture assessment, 1.9 cm-wide strips were removed from raw breast meat (2 strips per treatment) and three shear values were obtained for each strip (Lyon and Lyon, 1990; Zhang *et al.*, 2005). The samples were compressed with a V-cutting blade (guillotine), perpendicularly to muscle fiber orientation, at a compressing speed of 5 mm/s and a down stroke distance of 30 mm. The maximum resistance of the sample to shearing was recorded, corresponding to the highest peak of the curve (Ruiz de Huidobro *et al.*, 2005). The hardness of breast meat

obtained from chickens fed on the control and HC diets was evaluated in both trials, whereas the hardness of breast meat from chickens fed on the commercial diet was only evaluated in the second one.

2.7. Statistical analysis

ANOVA statistical tests and Tukey's honest significant difference (HSD) test for mean comparison were employed (JMP® Pro 12, SAS institute, 2015). Differences among means with $p < 0.05$ were accepted as representing statistically significant differences. For color analysis in breast and thigh muscles, the MIXED procedure was applied, including fixed effect of dietary treatments, feeding trial (as there were only two levels) and their interaction, and random effect of pens: $Y_{ijke} = Diet_i + Trial_j + Diet_i*Trial_j + Pen_k + \varepsilon_{ijke}$. For texture analysis in breast meat, a multi-factor analysis of variance was applied, including fixed effect of dietary treatments, type of storage, storage time and their interaction: $Y_{ijke} = Diet_i + Storage_j + Time_k + Diet_i*Storage_j + Diet_i*Time_k + Storage_j*Time_k + Diet_i*Storage_j*Time_k + \varepsilon_{ijke}$. A hypothesis test for a proportion was also performed using JMP® Pro 12 (SAS institute, 2015). Survival analysis was carried out with R statistical package (R project for statistical computing, 2014).

3. Results and discussion

3.1. Sensory and survival analyses

According to the sensory evaluation performed by the descriptive panel, the percentage of rejection was lower than 20% until day 10 for both breast and leg quarter meat. From then on, there was a continuous increase in the percentage of rejection, with a higher rejection of leg quarter meat than breast meat (Fig. 2). Breast meat is usually preferred rather than leg quarter meat (Elsner *et al.*, 1997). Despite the panelists did not know what chicken cut were evaluating, leg quarter meat was more rejected than breast meat probably due to a higher deterioration. After analyzing the results from the descriptive panel, it was planned the storage period for the samples analyzed by the consumer panel. However, a longer storage period was evaluated in the second trial (15 days) than in the first trial (11 days), as there were not many differences among samples in the first one, maybe due to the storage on consecutive days (6, 7, 8 and 9 days).

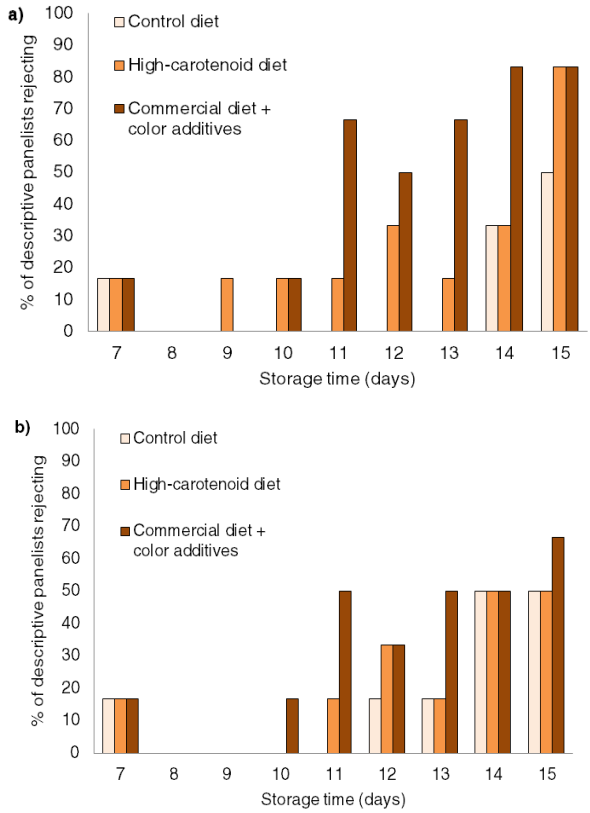
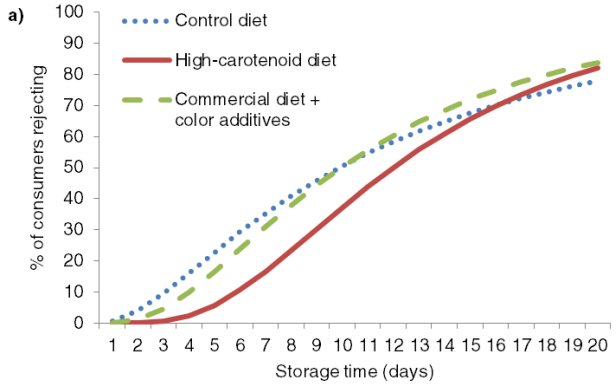
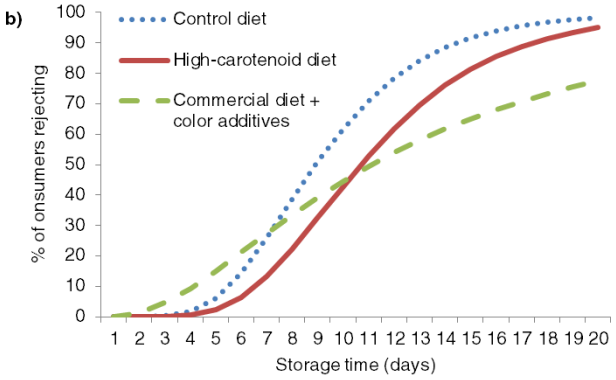


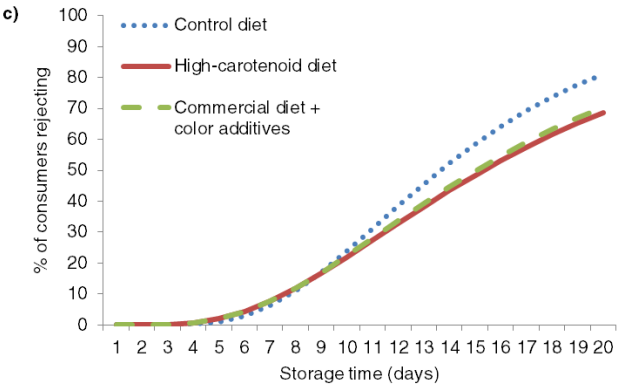
Figure 2. Percentage (%) of descriptive panelists rejecting meat samples obtained from chickens fed on the different diets and stored under refrigeration conditions during 15 days in the first trial. **a)** Breast meat; **b)** leg quarter meat.

The shelf life of chicken meat was defined as the time corresponding to a predefined percentage of rejection by consumers, traditionally 25 and 50% for foodstuffs (Giménez *et al.*, 2008; Hough *et al.*, 2003). In the present study, it was fixed at 25% (i.e. there is a 25% of probability that the consumer rejects the product) to be conservative and guarantee product quality since raw meat was evaluated. In the first trial, the shelf lives of meat from chickens fed on the control, HC and commercial diets were 5, 8 and 6 days for breasts, respectively, whereas for leg quarters shelf lives were 7, 8 and 6.5 days, respectively (Fig. 3). Nevertheless, in the second trial, breast meat samples showed a higher shelf life, whereas leg quarter meat samples showed a lower shelf life compared to the first trial (Fig. 3). Breast meat from chickens fed on the control, HC and commercial diets was rejected at 10

days by 25% of consumers. Leg quarter meat from chickens fed on the control and HC diets was rejected at 5.5 days and leg quarter meat from chickens fed on the commercial diet was rejected at 4.5 days by 25% of consumers.







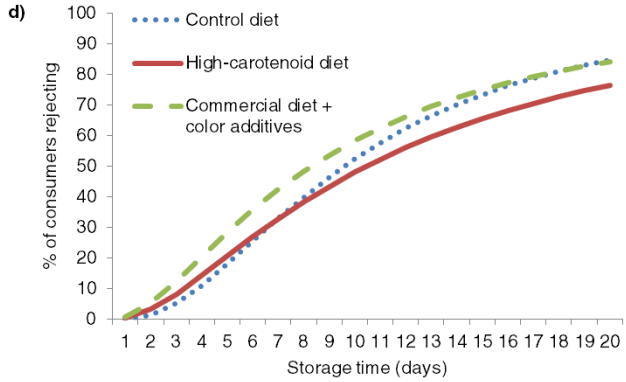


Figure 3. Percentage (%) of consumers rejecting meat samples obtained from chickens fed on the different diets in both trials. **a)** Breast meat (1st trial); **b)** leg quarter meat (1st trial); **c)** breast meat (2nd trial); **d)** leg quarter meat (2nd trial).

Consumers may not have been able to differentiate samples stored on consecutive days (6, 7, 8 and 9 days) in the first trial. Since the consumer acceptance seems to be influenced by the storage period, a longer separation time (2 days) among stored samples may facilitate the sensory evaluation. Leg quarter meat generally had a similar or lower shelf life compared to breast meat, which could be related to the deboning operation. Despite it was carried out under hygienic and refrigerated conditions, deboning always entails a higher manipulation, and therefore a higher risk of contamination. The survival analysis methodology was a useful tool to estimate the maximum storage time accepted by consumers, which could be established as 7–9 days. All chicken meat samples were statistically equally accepted after this period (Table 3).

Table 3. *p*-values corresponding to sensory analysis of breast and thigh meat at 7, 9, 11 and 15 days' storage. Samples are equally accepted if $p > 0.05$.

	Meat	Diet	*p*-value at day 7	*p*-value at day 9	*p*-value at day 11	*p*-value at day 15
1st trial	Breast (n = 69)	Control	0.500	0.500	0.168	-
		High-carotenoid	0.954	0.886	0.886	-
		Commercial + color additives	0.926	0.405	0.235	-
	Thigh (n = 45)	Control	1.000	0.068	0.008	-
		High-carotenoid	0.992	0.724	0.500	-
		Commercial + color additives	0.724	0.186	0.617	-
2nd trial	Breast (n = 58)	Control	1.000	1.000	0.997	0.044
		High-carotenoid	1.000	1.000	0.999	0.552
		Commercial + color additives	0.997	1.000	1.000	0.552
	Thigh (n = 65)	Control	0.959	0.772	0.161	< 0.001
		High-carotenoid	0.690	0.402	0.001	0.012
		Commercial + color additives	0.229	0.161	0.107	< 0.001

It has been demonstrated that chickens fed on carotenoid-enhanced diets did not substantially differ from chickens fed on the control diet in any of the criteria evaluated, except for color parameters. Redness and yellowness scores were higher in chickens fed on carotenoid-enhanced diets than in those animals fed on the control diet (Díaz-Gómez *et al.*, 2017). Moreover, this meat could have a longer shelf life, as carotenoids are well-known antioxidants which may counteract the lipid oxidation, an important factor in determining shelf life of foods (Davies, 2004; Stahl and Sies, 2003). Recently, it has been reported that natural astaxanthin (extracted from shrimp subproducts) used in refrigerated marinated chicken steaks, did not affect lipid oxidation and preserved the microbiological quality for at least one week (Abdelmalek *et al.*, 2016). Along the same line, the addition of fucoxanthin from algae improved the appearance and lipid stability of chicken meat, especially cooked breast meat (Sasaki *et al.*, 2008).

3.2. Microbiological and physical analyses

The results found in the sensory analyses were supported by the microbiological analyses. Spoilage in poultry meat usually occurs prior to any increase in the levels of pathogenic microorganisms (Alonso-Hernando *et al.*, 2012). Consequently, spoilage bacteria serve as indicators for microbiological shelf life.

Initial (day 1) counts were lower for *Pseudomonas* spp. than for total aerobic mesophilic bacteria in both refrigerated and thawed samples. In the first trial, microbial counts ranged from 0–10^2 and 10^3–10^4 cfu/g, for *Pseudomonas* and total aerobic mesophilic bacteria, respectively, in refrigerated samples, and from 10–10^3 and 10^4–10^5 cfu/g, for *Pseudomonas* and total aerobic mesophilic bacteria, respectively, in thawed samples (Table 4a).

Table 4. Microbial counts (cfu/g) in breast and thigh meat obtained from chickens fed on the different diets. **a)** First trial; **b)** second trial. R, refrigerated; T, thawed; Pse, *Pseudomonas* spp.; TAMB, total aerobic mesophilic bacteria.

a)				Day 1	Day 4	Day 7	Day 9
Control diet	Breast meat	R	Pse	10	10^2	10^2	10^3
		R	TAMB	10^3	10^5	10^6	10^5
		T	Pse	10	10^2	10^2	10^3
		T	TAMB	10^4	10^4	10^4	10^4
	Thigh meat	R	Pse	0	10^2	10^3	10^3
		R	TAMB	10^3	10^4	10^6	10^7
		T	Pse	10^3	10^2	10^5	10^6
		T	TAMB	10^5	10^5	10^5	10^6
High-carotenoid diet	Breast meat	R	Pse	10	10	10^3	10^5
		R	TAMB	10^4	10^5	10^5	10^7
		T	Pse	10	10^2	10^2	10^2
		T	TAMB	10^5	10^4	10^5	10^5
	Thigh meat	R	Pse	10^2	10^4	10^3	10^5
		R	TAMB	10^4	10^6	10^6	10^6
		T	Pse	10^3	10^3	10^4	10^3
		T	TAMB	10^4	10^5	10^6	10^7
Commercial diet + color additives	Breast meat	R	Pse	10	0	10^4	10^5
		R	TAMB	10^4	10^4	10^6	10^6
		T	Pse	10	10	10^2	10^2
		T	TAMB	10^4	10^4	10^4	10^5
	Thigh meat	R	Pse	0	10^2	10^4	10^6
		R	TAMB	10^4	10^5	10^6	10^6
		T	Pse	10^3	10^3	10^5	10^3
		T	TAMB	10^4	10^5	10^6	10^6

b)				Day 1	Day 4	Day 7	Day 9
Control diet	Breast meat	R	Pse	0	10	10^3	10^4
		R	TAMB	10^4	10^4	10^5	10^7
		T	Pse	10^2	10^2	10^2	10^5
		T	TAMB	10^4	10^4	10^4	10^6
	Thigh meat	R	Pse	0	10	10^4	10^4
		R	TAMB	10^5	10^5	10^6	10^6
		T	Pse	10^2	10^2	10^4	10^6
		T	TAMB	10^5	10^4	10^4	10^6
High-carotenoid diet	Breast meat	R	Pse	0	10^2	10^2	10^5
		R	TAMB	10^5	10^5	10^4	10^6
		T	Pse	10^3	10^4	10^4	10^7
		T	TAMB	10^4	10^4	10^5	10^7
	Thigh meat	R	Pse	10	10^2	10^3	10^5
		R	TAMB	10^5	10^5	10^6	10^6
		T	Pse	10^4	10^2	10^3	10^5
		T	TAMB	10^5	10^5	10^5	10^6
Commercial diet + color additives	Breast meat	R	Pse	10	10	10^3	10^4
		R	TAMB	10^4	10^4	10^5	10^5
		T	Pse	10^4	10^3	10^3	10^6
		T	TAMB	10^5	10^4	10^5	10^5
	Thigh meat	R	Pse	0	10^3	10^4	10^5
		R	TAMB	10^5	10^5	10^6	10^7
		T	Pse	10^5	10^5	10^5	10^6
		T	TAMB	10^5	10^5	10^5	10^6

In the second trial, microbial counts ranged from 0–10 and 10^4–10^5 cfu/g, for *Pseudomonas* and total aerobic mesophilic bacteria, respectively, in refrigerated samples, and from 10^2–10^5 and 10^4–10^5 cfu/g, for *Pseudomonas* and total aerobic mesophilic bacteria, respectively, in thawed samples (Table 4b).

In all cases, microbial counts increased progressively with storage time (from 1 to 9 days). Regardless the diet treatment, chicken meat samples reached the highest microbial counts at day 9 in both trials. In the first trial, microbial counts were not higher than 10^6 cfu/g for *Pseudomonas* spp. and not higher than 10^7 cfu/g for total aerobic mesophilic bacteria in both refrigerated and

thawed samples. In the second trial, microbial counts were not higher than 10^7 cfu/g for *Pseudomonas* spp. and not higher than 10^7 cfu/g for total aerobic mesophilic bacteria in both refrigerated and thawed samples.

Pseudomonas spp. are considered dominant spoilage bacteria in refrigerated poultry (Wang *et al.*, 2017). Their microbial counts increased proportionally with storage time, but the final counts were lower than in other studies (Wang *et al.*, 2017). Total aerobic microbial counts were within the limit of acceptability for poultry products of 10^7 cfu/g (Senter *et al.*, 2000). The limit value of 10^7 cfu/g was reached after approximately 9 days. Thus, this period is considered to be the microbiological shelf-life of the product. Microbiological quality was not evaluated after day 9, as counts for total aerobic mesophilic bacteria and *Pseudomonas* spp. had already reached 10^6–10^7 cfu/g.

Ultimate pH values are the main factor determining poultry meat color (Totosaus *et al.*, 2007). Chicken meat samples had similar pH values over 9 days of storage in both trials, with higher values in thigh meat than in breast meat. In the first trial, the pH of both refrigerated and thawed breast meat samples ranged from 5.9–6.3. However, pH values ranged from 6.4–6.9 and 6.1–6.6 in refrigerated and thawed thigh meat samples, respectively (Table 5a).

Table 5. pH values in breast and thigh meat obtained from chickens fed on the different diets. **a)** First trial; **b)** second trial. R, refrigerated; T, thawed.

a)			Day 1	Day 4	Day 7	Day 9
Control diet	Breast meat	R	5.9	6.1	5.9	5.9
		T	6.1	6.0	5.9	6.0
	Thigh meat	R	6.5	6.4	6.5	6.5
		T	6.6	6.2	6.2	6.2
High-carotenoid diet	Breast meat	R	5.9	5.9	5.9	6.0
		T	6.0	6.0	6.2	6.1
	Thigh meat	R	6.6	6.5	6.9	6.6
		T	6.4	6.5	6.3	6.6
Commercial diet + color additives	Breast meat	R	5.9	6.3	6.1	5.9
		T	6.1	6.0	6.1	6.2
	Thigh meat	R	6.7	6.6	6.7	6.6
		T	6.3	6.5	6.1	6.2

b)			Day 1	Day 4	Day 7	Day 9
Control diet	Breast meat	R	6.2	6.0	6.1	6.1
		T	6.1	6.1	5.9	6.1
	Thigh meat	R	6.5	6.1	6.3	6.5
		T	6.2	6.3	6.6	6.4
High-carotenoid diet	Breast meat	R	6.1	6.1	6.2	6.0
		T	6.0	5.9	6.1	6.0
	Thigh meat	R	6.3	6.1	6.4	6.5
		T	6.5	6.6	6.4	6.5
Commercial diet + color additives	Breast meat	R	6.1	5.9	6.2	6.1
		T	6.2	5.9	6.0	6.0
	Thigh meat	R	6.3	6.0	6.2	6.4
		T	6.6	6.6	6.8	6.4

In the second trial, the pH of both refrigerated and thawed breast meat ranged from 5.9–6.2, while it ranged from 6–6.5 and 6.2–6.8 in refrigerated and thawed thigh meat samples, respectively (Table 5b). In both trials, pH values were similar among meat samples obtained from chickens fed on the different diets. In accordance with Rathgeber *et al.* (1999), all our meat samples were obtained from muscles that had undergone normal glycolysis (pH > 5.8).

After slaughter, meat samples obtained from chickens fed on the HC and commercial diets had significantly higher redness and yellowness ($p < 0.05$) than those from chickens fed on the control diet (Table 6). In both trials, the differences found after slaughter remained during storage, either in refrigerated (Table 7) or in thawed samples (Table 8). In spite of not observing signs of color depletion, there was a slight variability among days. Color parameters could only be evaluated in two samples (breast and thigh meat) from each diet treatment on each day of storage due to the limited number of samples. Despite this, it was sufficient to ensure that the meat color was maintained during storage, either in refrigerated or frozen condition, and to corroborate the results obtained during slaughter and during the animal feeding trial, in which color evolution was individually measured in all animals (Díaz-Gómez *et al.*, 2017). HC maize has been shown to provide meat with a long-lasting color without the need to add color additives to the feed. Furthermore, in previous studies, chickens fed on the

HC diet accumulated more bioavailable carotenoids in peripheral tissues, muscle, skin and fat, than those fed on the control diet, e.g. chickens fed on the HC diet had similar levels of lutein and zeaxanthin in breast and thigh meat, 1 and 4 μg/g freeze-dried tissue, respectively, for breast meat, and 1 and 5 μg/g freeze-dried tissue, respectively, for thigh meat (Nogareda *et al.*, 2016).

Table 6. Color determination in breast and thigh muscles of chickens fed on the different diets after slaughter, using the CIELab trichromatic system to score lightness (L*), redness (a*) and yellowness (b*). The values shown are the mean and standard error (n = 24 in all the diets except for n = 22 in commercial diet in the first trial). Means within a row with no superscript in common are significantly different ($p < 0.05$). **a)** First trial; **b)** second trial.

a)		Control diet	High-carotenoid diet	Commercial diet + color additives
Breast muscle	L*	47.34 ± 0.08 ^{a}	45.71 ± 0.69 ^{a}	44.68 ± 0.64 ^{a}
	a*	0.06 ± 0.14 ^{b}	3.09 ± 0.18 ^{a}	3.52 ± 0.12 ^{a}
	b*	2.20 ± 0.24 ^{c}	7.05 ± 0.38 ^{b}	8.82 ± 0.42 ^{a}
Thigh muscle	L*	51.28 ± 0.83 ^{a}	49.20 ± 0.64 ^{ab}	48.21 ± 0.58 ^{b}
	a*	0.90 ± 0.16 ^{b}	4.37 ± 0.17 ^{a}	4.90 ± 0.17 ^{a}
	b*	3.28 ± 0.37 ^{c}	9.35 ± 0.49 ^{b}	11.78 ± 0.05 ^{a}
b)		Control diet	High-carotenoid diet	Commercial diet + color additives
Breast muscle	L*	44.43 ± 0.53 ^{a}	42.87 ± 0.44 ^{a}	43.77 ± 0.52 ^{a}
	a*	0.69 ± 0.13 ^{b}	3.43 ± 0.15 ^{a}	3.64 ± 0.20 ^{a}
	b*	2.70 ± 0.30 ^{c}	8.19 ± 0.34 ^{b}	10.43 ± 0.38 ^{a}
Thigh muscle	L*	51.97 ± 0.72 ^{a}	50.15 ± 0.44 ^{a}	51.83 ± 0.57 ^{a}
	a*	1.66 ±0.24 ^{b}	5.23 ± 0.25 ^{a}	4.80 ± 0.23 ^{a}
	b*	3.48 ± 0.37 ^{c}	10.68 ± 0.76 ^{b}	13.43 ± 0.60 ^{a}

Table 7. Color evolution in refrigerated samples of breast and thigh meat according to the CIELab trichromatic system as lightness (L*), redness (a*) and yellowness (b*). **a)** First trial; **b)** second trial.

a)			Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	Breast meat	L*	53.91	52.83	47.59	47	47.16
		a*	-0.87	-0.33	0.21	2.55	1.06
		b*	-1.05	1.87	5.67	7.7	2.59
	Thigh meat	L*	54.26	50.04	58.52	53.74	46.14
		a*	0.36	0.01	1.03	2.67	4.5
		b*	0.76	4.53	1.46	5.36	8.31
High-carotenoid diet	Breast meat	L*	48.22	45.77	47.22	44.3	54.63
		a*	4.93	4.14	4.79	4.86	1.32
		b*	11.21	9.13	12.92	14.03	3.91
	Thigh meat	L*	46.24	45.81	44.41	44.46	42.87
		a*	5.38	4.78	5.67	5.48	8.02
		b*	10.22	9.87	13.71	10.55	10.7
Commercial diet + color additives	Breast meat	L*	46.31	41.37	48.94	47.33	49.21
		a*	5.36	4.57	4.51	4.2	3.68
		b*	11.4	12.64	19.25	12.12	18.26
	Thigh meat	L*	50.52	49.94	50.17	51.7	49.75
		a*	2.4	3.12	2.35	4.32	5.45
		b*	2.18	9.37	14.7	19.51	19.71

b)			Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	Breast meat	L*	46.31	46.33	46.56	44.28	41.93
		a*	0.08	0.67	-0.26	2.15	2.57
		b*	0.84	5.56	0.92	4.2	4.61
	Thigh meat	L*	47.35	57.1	49.74	48.83	54.2
		a*	5.5	3.53	0.77	5.19	-0.14
		b*	13.05	4.73	3.21	4.97	6.37
High-carotenoid diet	Breast meat	L*	46.83	46.61	46.71	49.32	40.87
		a*	2.79	3.43	2.51	2.38	4.81
		b*	8.53	9.62	7.73	14.38	8.03
	Thigh meat	L*	47.35	48.85	48.45	52.49	50.67
		a*	5.5	4.15	4.17	4.61	5.28
		b*	13.05	12.97	11.86	9.7	3.99
Commercial diet + color additives	Breast meat	L*	44.33	48.58	43.07	45.2	46.73
		a*	3.53	3.39	4.76	2.84	2.64
		b*	13.11	12.24	14.36	11.45	11.89
	Thigh meat	L*	47.16	58.07	48.29	46.7	51.07
		a*	5.05	3.21	4.17	5.93	7.74
		b*	12.26	6.65	15.45	13.52	12.3

Table 8. Color evolution in thawed samples of breast and thigh meat according to the CIELab trichromatic system as lightness (L*), redness (a*) and yellowness (b*). **a)** First trial; **b)** second trial.

a)			Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	Breast meat	L*	41.93	49.35	48.75	45.64	47.39
		a*	4.23	3.16	1.5	1.2	0.31
		b*	5.28	6.27	5.13	4.19	6.59
	Thigh meat	L*	52.24	53.83	52.64	50.9	49
		a*	3.04	6.16	6.57	2.06	3.69
		b*	3.26	12.13	15.69	1.15	8.72
High-carotenoid diet	Breast meat	L*	44.32	45.28	48.65	43.98	43.58
		a*	6.03	4.03	2.26	5.13	3.96
		b*	11.26	9.83	8.71	14.16	11.22
	Thigh meat	L*	53.33	52.75	41.42	49.47	53.64
		a*	2.78	10.45	9.07	4.89	4.51
		b*	3.17	19.78	13.4	13.06	4.54
Commercial diet + color additives	Breast meat	L*	43.14	43.03	45.23	46.32	40.76
		a*	5.68	4.53	3.79	4.85	5.15
		b*	9.87	15.41	13.21	16.52	14.88
	Thigh meat	L*	51.67	41.69	47.03	59.17	55.13
		a*	7.72	9.16	7.89	3.3	5.35
		b*	7.38	17.17	21.39	11.06	7.7

b)			Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	Breast meat	L*	47.44	44.85	47.95	44.67	43.44
		a*	0.64	1.47	1.81	3.06	1.33
		b*	3.79	3.49	6.1	3.73	6.9
	Thigh meat	L*	49.53	48.91	51.81	44.31	49.77
		a*	5.14	3.53	1.93	5.12	2.76
		b*	8.4	9.73	8.59	5.64	9.15
High-carotenoid diet	Breast meat	L*	51.16	46.05	46.54	46.93	43.53
		a*	2.77	5.84	4.36	3.5	6.34
		b*	5	13.32	8.78	9.36	14.59
	Thigh meat	L*	41.25	47.99	45	46.38	44.96
		a*	9.58	8.14	7.35	7.35	9.26
		b*	14.02	16.94	16.64	16.68	15.63
Commercial diet + color additives	Breast meat	L*	47.14	49.34	45.47	42.63	46.47
		a*	3.12	4.28	4.74	7.35	3.81
		b*	9.68	13.85	10.53	17.01	13.75
	Thigh meat	L*	48.66	52.68	52.83	49.95	50.15
		a*	5.68	7.39	2.75	5.57	9.52
		b*	13.11	20.64	5.05	11.34	15.97

Regarding texture, the results obtained from each trial (Table 9) were analyzed separately because only two diet treatments (control and HC diets) were analyzed in the first trial whereas all the diet treatments (control, HC and commercial diets) were analyzed in the second one. Considering the different treatments, there were no significant differences ($p > 0.05$) in breast meat hardness between control and HC diets in the first trial. However, breast meat from chickens fed on the control diet had significantly lower hardness ($p < 0.05$) in the second trial. With respect to the type of storage, there were significant differences between thawed and refrigerated samples in the first trial ($p < 0.05$), but these differences were not observed in the second one ($p > 0.05$). The breast meat hardness increased from day 1 to day 11 in both trials, although this difference was only statistically significant in the second trial ($p < 0.05$). The slight differences found in both trials could be due to early deboning, which is thought to produce meat with a wide range of tenderness (Owens *et al.*, 2004). Breast meat obtained from the different diets could be classified as very tender (1.83–3.96 kg) in accordance with Xiong *et al.* (2006), although in our experiment firmness was evaluated in raw meat rather than cooked meat. Considering that cooking methods can

affect moisture and texture of the final product, as they increase hardness in meat (Owens *et al.*, 2004; Ruiz de Huidobro *et al.*, 2005), it would be necessary to evaluate the hardness in cooked breast meat in future studies.

Generally, freezing and thawing processes did not affect quality parameters evaluated in the present study. Regardless the type of storage, meat from chickens fed on the HC diet was similar to meat from chickens fed on the commercial diet supplemented with color additives in terms of sensory shelf life, physical parameters and microbial counts. However, it must be considered that yellowness scores were higher in chickens fed on the commercial diet supplemented with color additives compared to chickens fed on the HC diet due to the higher yellow xanthophyll levels from the marigold flower extract.

HC maize used as a feed component of poultry diet did not adversely affect any quality parameter evaluated and meat from chickens fed on the HC diet was equally accepted than meat from the other two diets until day 9. The shelf life of meat obtained from chickens fed on the HC diet, as measured in an acceptability test and corresponding to a 25% rejection probability, was approximately 8–10 and 5.5–8 days, for breast and leg quarter meat, respectively. Taking into account the results obtained in the survival and microbiological analyses, 7–9 days could be considered as the maximum storage period for this chicken meat accepted by consumers. This is supported by the physical parameters (pH, color and texture), which remained quite stable during storage. In further studies, it would be interesting to study the effect of carotenoids, as intrinsic compounds of HC maize, on lipid peroxidation of chicken meat.

Table 9. Texture parameter measured in breast meat and expressed as the maximum shear force (kg). Values shown are the mean and standard error (n = 6). Means within each trial with no superscript in common are significantly different ($p < 0.05$). **a)** First trial; **b)** second trial. R, refrigerated; T, thawed.

a)		Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	R	2.24 ± 0.05 ^{bc}	2.94 ± 0.09 ^{abc}	2.85 ± 0.19 ^{abc}	2.56 ± 0.20 ^{abc}	2.74 ± 0.27 ^{abc}
	T	2.96 ± 0.25 ^{abc}	2.67 ± 0.17 ^{abc}	3.18 ± 0.10 ^{ab}	3.26 ± 0.24 ^{a}	2.33 ± 0.14 ^{abc}
High-carotenoid diet	R	2.20 ± 0.11 ^{c}	2.85 ± 0.26 ^{abc}	3.06 ± 0.18 ^{abc}	2.87 ± 0.16 ^{abc}	2.88 ± 0.12 ^{abc}
	T	2.69 ± 0.16 ^{abc}	3.20 ± 0.13 ^{a}	2.85 ± 0.20 ^{abc}	3.17 ± 0.19 ^{ab}	2.65 ± 0.26 ^{abc}
b)		Day 1	Day 4	Day 7	Day 9	Day 11
Control diet	R	2.40 ± 0.17 ^{cd}	2.12 ± 0.28 ^{d}	2.38 ± 0.30 ^{cd}	2.97 ± 0.11 ^{abcd}	3.55 ± 0.22 ^{abc}
	T	2.48 ± 0.16 ^{cd}	2.35 ± 0.17 ^{cd}	2.47 ± 0.32 ^{cd}	2.61 ± 0.22 ^{cd}	2.87 ± 0.18 ^{abcd}
High-carotenoid diet	R	2.89 ± 0.07 ^{abcd}	2.88 ± 0.21 ^{abcd}	2.39 ± 0.26 ^{cd}	2.10 ± 0.21 ^{d}	3.96 ± 0.29 ^{a}
	T	3.58 ± 0.24 ^{abc}	2.89 ± 0.38 ^{abcd}	2.55 ± 0.24 ^{cd}	3.12 ± 0.24 ^{abcd}	3.03 ± 0.13 ^{abcd}
Commercial diet + color additives	R	2.77 ± 0.19 ^{abcd}	2.14 ± 0.10 ^{d}	2.52 ± 0.30 ^{cd}	2.00 ± 0.09 ^{d}	3.86 ± 0.28 ^{ab}
	T	2.74 ± 0.17 ^{abcd}	2.67 ± 0.22 ^{bcd}	3.18 ± 0.30 ^{abcd}	3.05 ± 0.27 ^{abcd}	2.79 ± 0.11 ^{abcd}

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CHAPTER V

Influence of cooking conditions on carotenoid content and stability in porridges prepared from high-carotenoid maize

Joana Díaz-Gómez, Antonio J. Ramos, Changfu Zhu, Olga Martín-Belloso and Robert Soliva-Fortuny

Plant Foods for Human Nutrition (2017), 72 (2): 113–119

Influence of cooking conditions on carotenoid content and stability in porridges prepared from high-carotenoid maize

Joana Díaz-Gómez [a], Antonio Javier Ramos [a], Changfu Zhu [b], Olga Martín-Belloso [a] and Robert Soliva-Fortuny [a]

[a] Food Technology Department, University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain;
[b] Plant and Forestry Science Department, University of Lleida–Agrotecnio Center, Av. Rovira Roure 191, 25198 Lleida, Spain

Abstract

Maize is a staple food crop in many developing countries, hence becoming an attractive target for biofortification programs toward populations at risk of micronutrient deficiencies. A South African white endosperm maize inbred line was engineered with a carotenogenic mini-pathway to generate high-carotenoid maize, which accumulates β-carotene, lutein and zeaxanthin. As maize porridge is a traditional meal for poor populations in sub-Saharan African countries, high-carotenoid maize was used as raw material to prepare different maize meals. The objective of this work was to assess the impact of popular home-cooking techniques and different cooking parameters (temperature, time and pH) on the final carotenoid content in the cooked product, using a spectrophotometric technique based on the mean absorption of carotenoids at 450 nm. Carotenoid levels were not only preserved, but also enhanced in high-carotenoid maize porridges. The carotenoid content was increased when temperatures ≤ 95 °C were combined with short cooking times (10–60 min). The most optimum thermal treatment was 75 °C/10 min. When treated under those conditions at pH 5, high-carotenoid maize porridges doubled the initial carotenoid content up to 88 μg/g dry weight. Regarding to cooking techniques, the highest carotenoid content was found when unfermented thin porridges were prepared (51 μg/g dry weight of high-carotenoid maize porridge). We conclude that high-carotenoid maize may contribute to enhance the dietary status of rural populations who depend on maize as a staple food.

Keywords

Carotenoids, processing, fermentation, biofortification, metabolic engineering

1. Introduction

Vitamin A deficiency (VAD) is a major public health problem worldwide, and particularly in African and South-East Asian countries. A poor diet can lead to chronic insufficient vitamin A intake, the main underlying cause of VAD, which may affect vision and cellular processes. Approximately, one-third of the world's pre-school-age children and up to 15% of pregnant women are estimated to be affected by VAD (WHO, 2009). Vitamin A is an essential nutrient which is converted into retinal in the retina of the eye, and it is also necessary for embryonic development and the maintenance of epithelial and inmune cells (Institute of Medicine, 2001). Meat and dairy products are common dietary sources of vitamin A, whereas dietary carotenoids (provitamin A –PVA–) are obtained from colored fruits and vegetables (Institute of Medicine, 2001). Maize is a staple food for more than a billion people worldwide, especially for populations in Central America and Southern Africa where this crop provides almost one-third of their caloric needs (Suri and Tanumihardjo, 2016). In sub-Sarahan African countries, white maize is mainly used to prepare maize meals, but it is devoid of carotenoids, which may contribute to the prevalence of VAD in these populations (Nuss and Tanumihardjo, 2010).

Carotenoids are isoprenoid compounds found in fruits and vegetables, either as hydrocarbon carotenes, or as oxygen-containing xanthophylls. Some carotenoids (such as β-carotene, α-carotene and β-cryptoxanthin) can contribute to human health because of their PVA activity, while others act as antioxidants (Britton, 1995). The intracellular location of carotenoids plays an important role in their bioavailability. In photosynthetic plant tissues, e.g. dark-green leafy vegetables and carrots, they are bound to proteins in the chloroplasts, whilst in non-photosynthetic plant tissues, e.g. yellow and orange fruits, they are mainly found in chromoplasts, dissolved in oil droplets (Ball, 2006). Maize carotenoids are also present in substantial levels in amyloplasts, plastids specialized for storage of starch granules (Howitt and Pogson, 2006).

Maize is usually consumed as porridge, cooked either with a thin or thick consistency by rural populations in sub-Saharan African countries. Some maize products are fermented to facilitate grain processing and to avoid bacterial contamination, but the use of this technique depends on the geographical localization (De Groote and Kimenju, 2012). The cooking technique and cooking conditions (time and temperature) may either increase or decrease the carotenoid concentration, e.g. stewing increases it while frying reduces it (Murador *et al.*, 2014). Thermal treatments inactivate oxidative enzymes and disrupt some cellular structures. Thus, higher carotenoid content in food products is usually reported under mild thermal conditions. The unsaturated structure of carotenoids makes them susceptible to degradation under high temperature, low pH, light and reactive oxygen species. Processing and storage may favor the exposure to oxygen and oxidative enzymes, thus causing structural modifications in carotenoids such as geometric isomerization and oxidation (Borsarelli and Mercadante, 2010).

Biofortification of staple crops with organic nutrients is an alternative long-term strategy to improve nutritional health of populations at risk of micronutrient deficiencies. It can be performed at source (i.e. in the plant itself) through agronomic interventions, plant breeding or genetic engineering (Gómez-Galera *et al.*, 2010). Several staple crops have been genetically engineered to enhance carotenoid levels and composition. Namely, high-carotenoid (HC) maize which was engineered to accumulate high levels of β-carotene, lutein and zeaxanthin (Zhu *et al.*, 2008). The South African white maize inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase 1 (necessary for the biosynthesis of carotenoids), was used as a basis to create the HC maize through the introduction of two genes, *Zmpsy1* (*Zea mays* phytoene synthase 1) and *PacrtI* (*Pantoea ananatis* phytoene desaturase), under the control of endosperm-specific promoters (Zhu *et al.*, 2008). Recent studies have demonstrated a high carotenoid retention in biofortified crops after processing, either in transgenic biofortified crops such as sorghum and cassava, or in conventional biofortified crops such as maize, cassava and pumpkin (Díaz-Gómez *et al.*, 2017).

Different processing methods lead to substantial changes in the nutritional composition of maize products (Suri and Tanumihardjo, 2016). Thus, it is important to assess the effect of processing on novel maize varieties. Our objectives were: a) to investigate the effect of temperature, cooking time and pH on carotenoid content of HC maize; b) to evaluate the effect of different

home-cooking techniques on the final carotenoid content in maize porridges obtained from HC maize and its near isogenic line.

2. Materials and methods

2.1. Maize

The M37W white maize and its engineered HC maize derivative were sown in a location (Lleida, Catalonia) in the Northeast of Spain. The experimental field was approved by the Spanish Ministry of Economy and Competitiveness and the Catalan Government (B/ES/13/16 and B/ES/14/04). Maize cobs were harvested, dried at low temperature (35 ºC) for 24 h in a drying chamber, threshed and then kernels were milled (Ras® Mill, Romer® Labs Inc., MO, USA). The resulting flour was frozen at –18 °C until cooking and analysis. The fermented flour was prepared before cooking by adding 200 mL of tap water to 600 g of dry maize flour. The wet flour was allowed to spontaneously ferment in a pot with a lid at 30 °C in a heater for 48 h.

2.2. Maize porridge

Two independent experiments were carried out in order to assess the stability of carotenoids in HC maize under cooking conditions. Experiment 1 was performed to evaluate the effect of different processing parameters (temperature, time and pH) on the total carotenoid content (TCC) of HC maize. Results from this experiment were used to select the best processing conditions to perform Experiment 2, whose aim was to evaluate the effect of home-cooking techniques on the TCC of HC maize and its near isogenic line.

2.3. Experiment 1: evaluation of temperature, time and pH during processing

Thermal treatments from 75 to 95 °C were applied to allow gelatinization of maize starch, as this has been reported to occur at 65–76 °C (Morales-Sanchez *et al.*, 2009). Temperatures below 75 °C were discarded as gelatinization of maize starch did not occur. Maize porridges were prepared with 12.5 g of maize flour and 37.5 mL of aquous solution at different pH (4, 5 and 6). The mixture of maize flour and tap water had a pH ~5. Buffers were added to achieve pH 4 and pH 6. Citric acid 0.1 M (SAFC, Sigma-

Aldrich, Madrid, Spain) and di-sodium hydrogen phosphate 0.2 M (Scharlau, Scharlab, Barcelona, Spain) were used to prepare the buffer solutions following Sigma-Aldrich guidelines. Samples were packaged in polypropylene bags (Tecnopack®, ILPRA System, Barcelona, Spain) which were heat-sealed with no headspace just before the thermal treatments. Porridge samples were treated at different temperatures (75, 85 and 95 °C) using a water thermostatic bath (Microprocessor Control MPC, Huber, Offenburg, Germany). During thermal processing, samples were taken at 10, 20, 40, 60 and 120 min, and quickly cooled in an ice bath. Maize starch gelatinization took place under any tested combination of time and temperature. All the samples were processed in duplicate and frozen at −18 °C until carotenoid analysis.

2.4. Experiment 2: evaluation of home-cooking techniques

Unfermented and fermented flours of M37W and HC maize were used to prepare thin or thick maize porridges following a similar process to the cold-water procedure described by Li *et al.* (2007). Taking into account the initial moisture content of maize flours, a different volume of tap water was added to each porridge in order to produce equivalent final moisture content in the unfermented and fermented porridges after cooking. Therefore, four different types of porridge (unfermented thin porridge, fermented thin porridge, unfermented thick porridge and fermented thick porridge) were prepared in duplicate. The porridges compositions are summarized in Table 1.

Table 1. Composition of porridges.

Fermentation	Type of porridge	Maize flour (g)	Water (mL)
Unfermented	Thin	76.8	250
	Thick	125	150
Fermented	Thin	125 *	275
	Thick	125 *	80

* Fermented flour

Based on the preliminary results from experiment 1, a temperature of 95 °C combined with shorter cooking times was selected for this experiment. Porridges were cooked from 25 to 95 °C while stirring continously with a wood spoon. The temperature was monitored using an electronic

thermometer (IKA®, Staufen, Germany). It took 9 and 12 min to cook the thick and thin porridges, respectively. After cooking, they were left to cool down at room temperature for 2–3 hours. Moisture content and pH were determined in maize flour and porridge samples. All samples were processed in duplicate and frozen at −18 °C until carotenoid analysis.

2.5. Moisture measurement

The moisture content of the samples was determined by oven drying according to the Association of Official Analytical Chemists method (AOAC International, 2005).

2.6. pH measurement

The pH determination was performed using a Crison pH-meter combined with an electrode 52/32 (Hach Lange Spain, Barcelona, Spain).

2.7. Carotenoids analysis

Carotenoids extraction was carried out by mixing 0.5 g of samples with 15 mL of methanol:ethyl acetate (60: 40, *v/v*) and heating in a double boiler at 40 °C for 40 min using a hot plate equipped with electronic contact thermometer (IKA®, Staufen, Germany). Lipophilic compounds were then partitioned into 15 mL hexane:diethyl ether (90: 10, *v/v*) and the upper phase was collected (Rivera and Canela, 2012). Methanol, ethyl acetate and hexane were obtained from Scharlau Chemicals (Scharlab, Barcelona, Spain) and diethyl ether (99.5% with BHT) was obtained from Acros Organics (Fisher Scientific, Madrid, Spain). Total carotenoids were quantified with a CECIL 2021 UV/VIS spectrophotometer (Cecil Instruments, Cambridge, UK) by measuring the absorbance at 450 nm. The TCC was calculated using the following equation (Gross, 1991):

$$\text{TCC (µg carotenoids/g)} = \frac{\text{Abs x V x } 10^4}{A^{1\%}_{1\,cm} \text{ x W}}$$

where $A^{1\%}_{1\,cm}$ = specific absorbance or extinction coefficient, defined as the theoretical absorbance of a 1% solution (*w/v*) in a cuvette with a path length of 1 cm. An arbitrary $A^{1\%}_{1\,cm}$ of 2500 is used for the determination of total carotenoids. Abs = absorbance measured at 450 nm. V = total volume (mL). W = weight of the sample (g). 10^4 = conversion factor to obtain the concentration in units µg/g.

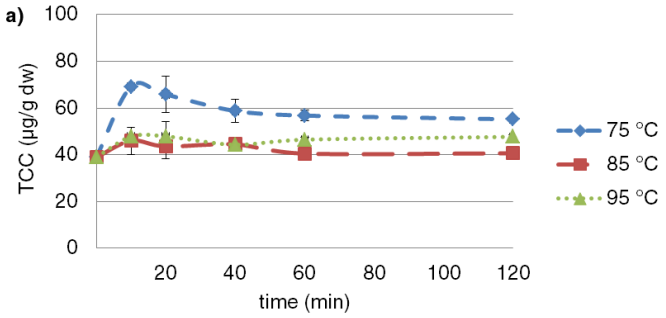
2.8. Statistical analysis

ANOVA and Tukey HSD test for means comparison were used (JMP® Pro 12 SAS institute, 2015). Differences among means with $p \leq 0.05$ were accepted as representing statistically significant differences.

3. Results and discussion

3.1. Influence of pH and temperature-time processing conditions on carotenoid content

The moisture content of HC maize porridges was 79.32% and the pH did not change during thermal processing time. The TCC in HC untreated maize porridges was 39 ± 1.3 μg/g dw (dry weight basis). The carotenoid content was higher in HC maize porridges after thermal processing than in uncooked porridges, varying according to the conditions used, although the increase was lower when long cooking times were applied (Fig. 1). There were statistically significant differences ($p < 0.05$) among treatments, as well as among temperature-time and temperature-pH interactions. The highest TCC was found when the lowest temperature was used (75 ºC), but there were no significant differences among samples treated at 85 and 95 °C. At pH 5, the carotenoid content of HC maize porridges heated at 75, 85 and 95 ºC for 10 min, was increased by 2.3–, 1.4– and 1.3–fold, respectively. At high temperatures (> 80 °C), most maize proteins are denaturated and unsolubilized (Sánchez del Angel *et al.*, 2003). Under those circumstances, carotenoids could not be easily released from the carotenoid-protein complexes, which may explain the differences found in carotenoid content between samples treated at 75 °C and 85−95 °C.



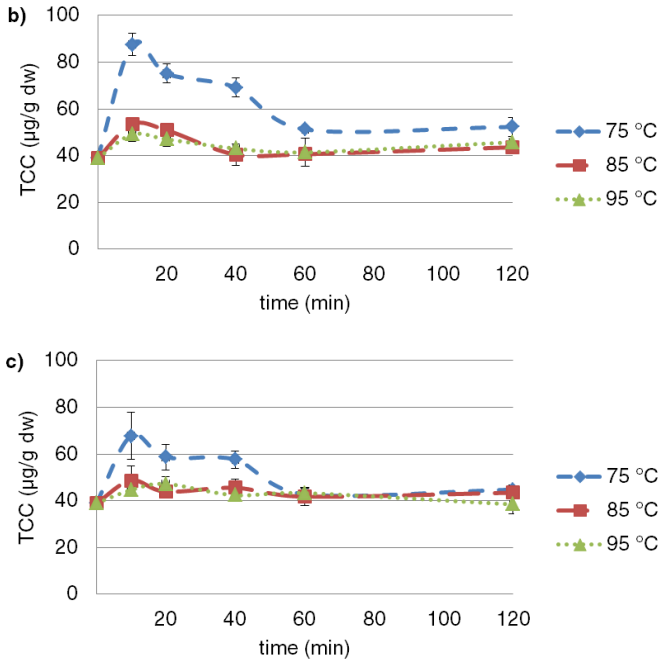


Figure 1. Total carotenoid content (TCC, in µg/g dw –dry weight basis–) in HC maize porridge treated at different processing conditions. Values are expressed as mean ± standard error (n = 2). **a)** pH 6; **b)** pH 5; **c)** pH 4.

With respect to time, the highest TCC was found in samples treated only for 10 min, with the prolongation of the treatment, the increase in TCC was consecutively lower. A slight decrease (0.5 µg/g dw) was only observed in HC porridge samples treated at 95 °C for 120 min. At pH 5, the carotenoid content of HC maize porridges heated at 75 ºC for 10, 20, 40, 60 and 120 min was increased by 2.25–, 1.93–, 1.77–, 1.32– and 1.34–fold, respectively. The best results were obtained in porridges treated at 75 °C during 10−40 min. When samples were treated for more than 40 min, no significant differences in carotenoid content were observed among treatments with respect to the temperature/time binomious. This may be explained by an increase in oxidation processes as longer cooking times increase oxidation due to excessive exposure to heat conditions (Murador *et al.*, 2014). Oxidation is the main factor accounting for carotenoid losses, and it is stimulated by light and heat, thus depending on available oxygen and the type of carotenoid (Ötles and Çagindi, 2008). Despite PVA carotenoids were not individually analyzed, it has been previously reported that their retention decreases with long times and high temperatures regardless of the cooking technique used (Rodriguez-Amaya, 1997). For instance, β-carotene was

generally reduced in orange-fleshed sweet potato after thermal treatment (e.g. boiling, frying, roasting and steaming), although there were slight differences among cultivars (Donado-Pestana *et al.*, 2012). As other biofortified crops, HC maize may prevent the loss of carotenoids during cooking more effectively than standard fortification, probably due to food matrix related effects (Díaz-Gómez *et al.*, 2017).

Temperature and processing time seem to be more important factors than pH in relation to the TCC in HC maize porridges. HC maize porridges heated at 75 ºC for 10 min had 1.3–fold higher carotenoid levels at pH 5 than at pH 6 and pH 4. This is a relevant isssue as the highest contents were obtained when tap water was used instead of buffer solutions. The lower TCC in samples at pH 6 may be related to a higher lipoxygenase activity during the first minutes of heat treatment. It has been previously reported that this enzyme exhibits its highest activity at pH 6 and it has a relatively high thermal stability, as it is inactivated at 93 °C/4−9 min depending on the maize cultivar (Barrett *et al.*, 2000; Niu *et al.*, 2015). On the other hand, the lower TCC in samples at pH 4 could be explained by protein denaturation and/or production of ion-pairs, as carotenoids exposed to acids are protonated, undergoing cis–trans isomerization and additional degradation reactions (Andrés-Bello *et al.*, 2013).

3.2. Effect of home-cooking techniques on carotenoid content

After performing the first experiment, a temperature of 95 °C and a cooking time below 20 min were selected for processing the maize under home conditions. The moisture content in thin porridges was significantly higher than in thick porridges ($p < 0.05$). A slightly higher moisture content was observed in fermented porridges regardless the amount of added water ($p < 0.05$). The differences found among porridges obtained from M37W and HC maize were not related to the type of maize (Table 2). The TCC was measured in the different porridges before and after cooking (Fig. 2). The TCC in the M37W and HC untreated maize porridges was 1.5 ± 0.5 and 39.5 ± 2.6 μg/g dw, respectively, in the unfermented porridges, and 2.7 ± 0.0 and 40 ± 0.6 μg/g dw, respectively, in the fermented porridges. The great difference in carotenoid content among M37W and HC maize porridges still existed after processing ($p < 0.001$). Thus, statistical analyses were performed separately depending on the type of maize. When unfermented flour was used, thin and thick porridges elaborated with HC maize had 9.2– and 15.6–

fold more carotenoids, respectively, than the same porridges based on M37W maize. This difference was higher when fermented flour was used. Thin and thick porridges elaborated with fermented flour from HC maize had 20.9– and 30–fold more carotenoids, respectively, than the same porridges based on M37W maize.

Table 2. Moisture content (%) in cooked porridge samples. Values are expressed as mean ± standard error (n = 4). Means with no letter in common are significantly different ($p < 0.05$).

	M37W	HC
Thin, unfermented	74.66 ± 0.38 ^{b}	76.64 ± 0.36 ^{ab}
Thin, fermented	78.26 ± 0.32 ^{a}	77.52 ± 0.62 ^{ab}
Thick, unfermented	60.61 ± 0.33 ^{c}	59.96 ± 0.70 ^{c}
Thick, fermented	61.52 ± 1.25 ^{c}	62.41 ± 1.02 ^{c}

The results obtained under laboratory conditions were confirmed, a significantly higher carotenoid content was found when HC maize flour (unfermented and fermented) was cooked into thin and thick porridges ($p < 0.05$). Our results are in accordance with those reported by Muzhingi *et al.* (2008) who found an increase in carotenoid concentration when yellow maize was cooked into porridge (either with a thin or thick consistency) at 100 °C for 30 min. Thin and thick porridges showed a higher retention of PVA carotenoids (106 to 131%) compared to *samp* porridge (53 to 98%), which uses dehulled maize kernels (Mugode *et al.*, 2014). Other authors found that *phutu* (a very thick porridge) and *samp* had a higher retention of PVA carotenoids (78 to 118%) compared to thin porridge (63 to 78%) (Pillay *et al.*, 2014). In both studies, the carotenoid retention was high in most cases (> 75%) (Mugode *et al.*, 2014; Pillay *et al.*, 2014). In our experiment, the highest increase in the final carotenoid content was found in thin porridges elaborated with unfermented flour for both maize types ($p < 0.05$) (Fig. 2). Thus, this technique would be the most adequate to cook maize into porridge in order to preserve its initial carotenoid content. In contrast, yellow maize porridge retained only 52% of total carotenoids (Kean *et al.*, 2008) and β-carotene retention losses were found when a biofortified β-carotene maize was cooked into porridge (24.8 and 24.5% of cumulative losses for unfermented and fermented porridges, respectively) (Li *et al.*, 2007).

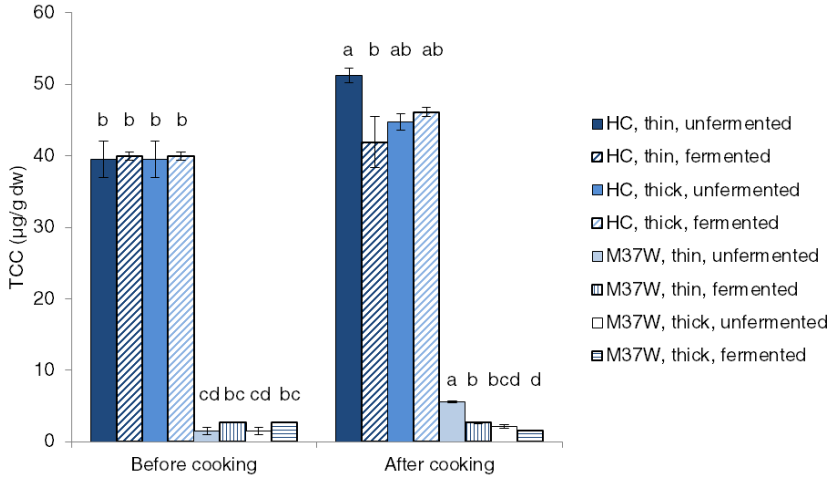


Figure 2. Total carotenoid content (TCC, in µg/g dw –dry weight basis–) in M37W and HC maize porridges measured before cooking (n = 2) and after cooking (n = 4). Thick and thin porridges were cooked at 95 °C during 9 and 12 min, respectively. Values are expressed as mean ± standard error. Means with no letter in common (within each type of maize) are significantly different ($p < 0.05$).

Fermentation did not affect carotenoid content of HC maize porridges, whereas M37W maize porridges doubled the initial carotenoid content after fermentation, which could be attributed to the enzimatic degradation of subcellular compartments. However, it seems that carotenoids were more negatively affected by heating in fermented flours, as carotenoid content was not so much increased (HC porridge) or even reduced (M37W porridge) when fermented flour was used. The wet flour was allowed to spontaneously ferment, resulting in the expected reduction of pH in both thin and think porridges, with significant differences among porridges ($p < 0.05$). The pH reduction was less prominent in HC fermented porridges than in M37W fermented porridges (Table 3), which may explain why carotenoid levels were not decreased when HC fermented flour was used. Li *et al.* (2007) showed that fermentation significantly affected the retention of β-carotene (10.2% of losses), although they did not find differences between unfermented and fermented porridges after cooking. The pH of their fermented maize flour was 4, whereas the minimum pH found in our porridge samples was ~ 5.

Table 3. pH values in cooked porridge samples. Values are expressed as mean ± standard error (n = 4). Means with no letter in common are significantly different ($p < 0.05$).

	M37W	HC
Thin, unfermented	6.20 ± 0.01 [a]	6.16 ± 0.02 [a]
Thin, fermented	4.98 ± 0.12 [b]	5.84 ± 0.06 [a]
Thick, unfermented	5.97 ± 0.01 [a]	5.97 ± 0.01 [a]
Thick, fermented	5.23 ± 0.23 [b]	5.79 ± 0.08 [a]

Our results are consistent with previous studies suggesting that carotenoids are released from the food matrix when thermal treatments are applied. Thermal processing can produce structural changes in food matrices, softening the plant tissue and disrupting the carotenoid-protein complexes, which leads to increased carotenoid levels in foods (Mercadante, 2008). In addition to thermal processing, food structure and dietary components (e.g. lipids) are other important factors to be considered in the carotenoid bioaccessibility (Saini *et al.*, 2015). On the other hand, processing may affect the stability of carotenoids as they are prone to isomerization and oxidation (Rodriguez-Amaya, 1997). Isomerization is one of the major consequences of food thermal processing on carotenoids, consequently, carotenoids bioavailability and physiological activity are affected (Mercadante, 2008). Therefore, it would be interesting to study the isomerization as well as the oxidation of HC maize after cooking in future studies. It must be kept in mind that carotenoid analysis has been performed using spectrophotometry instead of high-performance liquid chromatography (HPLC), which allows individual carotenoid detection and quantification. Spectrophotometry has been generally used to quantify total carotenoids in maize (Ndolo and Beta, 2013). When different spectrophotometric methods were compared to HPLC, the method based on the mean absorption of carotenoids at 450 nm was highly correlated with HPLC (Biehler *et al.*, 2010). Thus, it was selected as the most appropriate technique to screen, for the first time, the effect of cooking on HC maize. However, it has some limitations such as food with an unbalanced carotenoid profile, overestimation due to minor compounds and degradation products or underestimation due to colorless carotenoids (Biehler *et al.*, 2010). Further studies using HPLC will be necessary to assess the effect on PVA carotenoids and other nutritionally important carotenoids and, consequently, their role in human health.

Furthermore, white maize is preferred by many African consumers, as yellow maize is commonly associated with food aid and animal feed (Birol *et al.*,

2015). Nevertheless, it has been demonstrated that existing preferences for white maize or negative connotations associated with yellow maize do not adversely affect the acceptance of orange maize (PVA-biofortified maize) (Meenakshi *et al.*, 2012). Moreover, recent studies have shown that rural consumers are willing to pay for biofortified crops with visible traits (e.g. orange maize) as much as, if not more, than conventional varieties (Birol *et al.*, 2015). Therefore, education programs and information campaigns are necessary to promote the nutritional qualities of biofortified maize.

High-carotenoid maize can be a cost-effective and sustainable source of carotenoids, which may contribute to improve health status of populations who depend on maize as a staple crop. Our results show that final carotenoid content in maize meals varies depending on the processing conditions and cooking method applied, even it can be enhanced due to the increase in carotenoid extractability from the food matrix. The optimum thermal treatment for HC maize was 75 °C/10 min, followed by 75 °C/20 min and 75 °C/40 min. Higher temperatures are used when maize is cooked into porridge (95 °C), but they would also be acceptable, as carotenoid content was only decreased when cooking was prolonged during 2 hours. The carotenoid levels in maize meals may be affected by low pH values, thus, it would be useful to control pH levels in fermented products. Further studies are necessary to evaluate structural changes in carotenoids in HC maize after cooking, as well as, carotenoid bioavailability after consumption of meals based on HC maize.

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GENERAL DISCUSSION

High-carotenoid (HC) maize is a transgenic variety that accumulates high levels of carotenoids, including those with vitamin A activity. HC maize was developed to improve the nutritional health of human populations whose diet predominantly relies on cereals, with little access to fresh fruit and vegetables. However, it is necessary to evaluate its safety, agronomic performance and nutritional sufficiency before fulfilling its humanitarian objectives. HC maize has previously been shown to be substantially equivalent to its near isogenic line in terms of compositional analysis (except for the intended modification of the carotenoid content), to be safe in a 90–day sub-chronic toxicity study in mice and to have a similar agronomic performance to its near isogenic line (Arjó *et al.*, 2012; Zanga *et al.*, 2016a). A multidisciplinary project (VitaMaize: high quality and safe food through antioxidant fortified maize) was developed to carry out all these evaluations. This Doctoral Thesis is framed within that project and aims to evaluate the use of HC maize as feed and food component, through different analyses performed from the field to the fork. An overview of the main results obtained is presented and discussed in this section.

1. High-carotenoid maize grown under field conditions

Novel crops created with enhanced traits should be tested against their near isogenic lines under different environmental conditions to ensure that the novel traits (e.g. the accumulation of carotenoids) are preserved after field performance. Field testing is also necessary to assess the impact of abiotic (e.g. temperature, light, humidity and weather conditions) and biotic (e.g. pests and diseases) factors on crop performance (Zhu *et al.*, 2017). Maize fields are commonly colonized by several spoilage fungi and, consequently, a large variety of mycotoxins can be found. Thus, it is necessary to evaluate the susceptibility to fungal infection and mycotoxin contamination in novel maize varieties.

During three consecutive harvest seasons (2013, 2014 and 2015), the HC maize and its near isogenic line (the wild-type white endosperm variety M37W), were cultivated in an experiment field located in the University of Lleida (41°37'50"N, 0°35'27"E, 180 m). Both varieties were tested for the prevalence of fungal infection and the accumulation of FBs and AFs (Chapter I). There were only slight differences in fungal infection (expressed as the proportion of infected grains) between both types of maize. The dominant genus in all three harvest seasons was *Fusarium*. Maize kernels were

predominantly infected by *F. verticillioides* and, to a lesser extent, by *F. proliferatum*. These results are in accordance with previous studies in the Mediterranean area, which showed that *F. verticillioides* is the most prevalent fungus associated with maize in Southern Europe (Marín *et al.*, 2012). Despite the different climatic conditions between Northeastern and Northwestern Spain, *F. verticillioides* was also the predominant species in maize kernels grown in different locations in Northwestern Spain (Aguín *et al.*, 2014; Cao *et al.*, 2013).

According to Marín *et al.* (2004), *F. verticillioides* and *F. proliferatum* are able to germinate at 5–37 °C, 25 °C being the optimum temperature. These species are able to germinate within 24 h at 0.98–0.99 a_w at 25 °C, 0.88 being the minimum a_w for germination at that temperature. Hence, *F. verticillioides* and *F. proliferatum* are able to grow at 4–37 °C, but a_w range for growth is narrower than that for germination (≥ 0.90 a_w). However, the temperature and a_w conditions that allow fumonisin production are not necessarily the same as those that allow fungal germination or growth (Marín *et al.*, 2004). Generally, fumonisin-producing fungi need a medium temperature (i.e. 20–30 °C) and a a_w (i.e. 0.98 a_w) to produce the mycotoxin (Soriano and Dragacci, 2004). The optimum temperature for FB_1 production has been established at 20–30 °C and 15 °C, for *F. verticillioides* and *F. proliferatum*, respectively (Marín *et al.*, 1999). Figure 1 shows the different isopleths (i.e. lines drawn through all points of equal value for some measurable feature) for germination, growth and FB_1 production for the main fungi found in our experimental field.

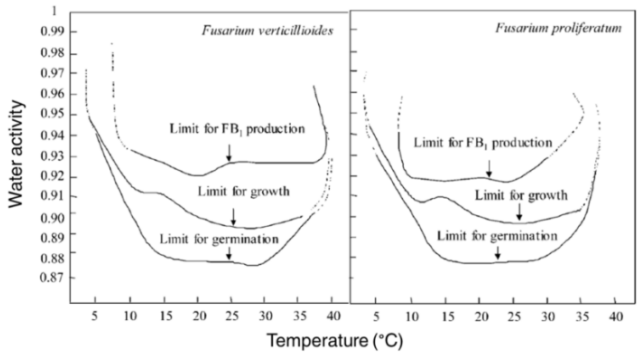

Figure 1. Isopleths showing the combined values of a_w and temperature that limit FB_1 production (1 mg/g), growth (0.1 mm/day) and germination (10% conidia) of isolates of *F. verticillioides* and *F. proliferatum*. Adapted from Marín *et al.* (2004).

Aspergillus spp. can occur in the field or as a storage mold in Mediterranean countries, but in our experimental field there were no *Aspergillus* species identified in any of the samples tested, and accordingly there was no contamination with AFs. To analyze fungal infection, two mediums were used, DRBC (dichloran rose-bengal chloramphenicol agar media) and DG18 (dichloran 18% glycerol agar media), but the results from the latter were finally discarded. DG18 media performs well to enumerate xerophilic molds, such as *Aspergillus* spp. due to its lower water activity (0.95 a_w) compared to DRBC media (> 0.95 a_w) (Hocking and Pitt, 1980; ISO, 2008a, 2008b; King *et al.*, 1979). The same procedure described in Chapter I was used: decontaminated kernels were plated in groups of five in DG18 Petri plates, and the plates were incubated for 7 days at 25 °C in darkness. After that, colonies of developing fungi were isolated in PDA (potato dextrose agar media), and the plates were incubated for 7 days at 25 °C in darkness. Molecular biology techniques were used to identify the fungal species. According to the results obtained using DG18 (Fig. 2), control maize (the wild-type variety M37W) was contaminated 26, 44 and 80% in 2013, 2014 and 2015, respectively, whereas HC maize was contaminated 54, 58 and 84% in 2013, 2014 and 2015, respectively.

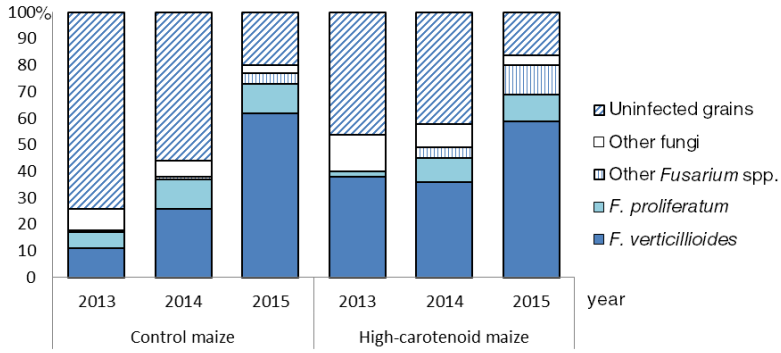

Figure 2. Mycobiota identification using DG18 media in control and high-carotenoid maize during the trials.

The results obtained from this media were not taken into account because *Fusarium* was the dominant genus, and DG18 is not such an adequate media for this genus. Regardless of the incubation time, DRBC performs better than other media to enumerate foodborne yeasts and molds, thus recovering higher numbers of fungi (Beuchat and Mann, 2016). Therefore, the results obtained using the DRBC media were used for the fungal infection and fungal identification.

Fusarium spp. infected most maize kernels; subsequently, fumonisin contamination was found in both maize varieties in all the years of study (Fig. 3), but the proportion of contaminated grains was substantially higher in the control maize (1.4–fold, 2.4–fold and 2–fold more in the 2013, 2014 and 2015 harvest seasons, respectively). A higher fumonisin contamination was found in both maize varieties in the second harvest, which was associated with higher rainfall two months before harvest, corresponding with an optimum period for fumonisin production and/or accumulation (Marín *et al.*, 2004).

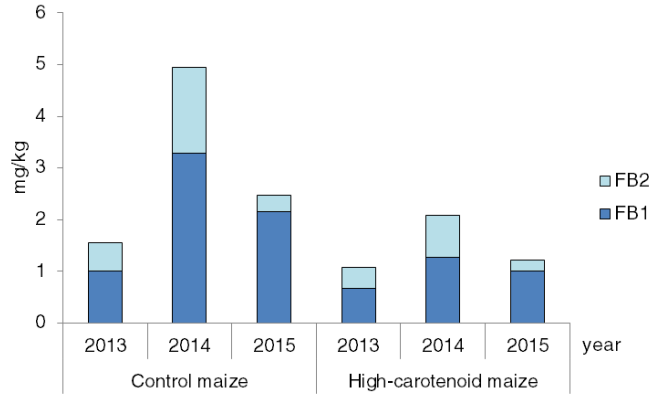

Figure 3. Fumonisin levels (FB_1 + FB_2, in mg/kg) in control and high-carotenoid maize during the trials. Values are means (n = 3).

The agronomic performance of HC maize and its near isogenic line under field conditions was also evaluated by researchers from our university, concluding that HC maize did not differ from its counterpart in terms of agronomic performance, particularly grain yield and its main components. Complementarily, two different fertilization regimes were evaluated: 0 and 200 kg N/ha, the latter applied as urea at the V6 stage (for a better understanding of maize growth stages see pages 15–16). The response to N was similar for both types of maize, indicating that the transgenes introduced do not influence the manner in which maize plants respond to N availability (Zanga *et al.*, 2016a). The application of N fertilizers, which lead to longer vegetative growth and higher leaf expansion, could extend the favorable conditions (i.e. high moisture levels from silking to maturation) that promote fungal infection (Blandino *et al.*, 2008). High and low rates of N fertilization have been related to higher fumonisin levels in maize fields, recommending a balanced N fertilization (200 kg N/ha) to ensure lower fumonisin

contamination (Blandino *et al.*, 2008). Spanish researchers only found a weak correlation between N fertilization (150–336 kg N/ha) and fumonisin levels in maize (Ariño *et al.*, 2009), whereas Italian researchers showed a significant increase in fumonisin levels in fertilized maize fields (270 kg N/ha) compared to those unfertilized in 2 of 3 years of study (Marocco *et al.*, 2008). However, these Italian researchers did not find significant differences in fumonisin levels between maize fields fertilized at 300 kg N/ha or without fertilization (Marocco *et al.*, 2009). Thus, the effect of N fertilization on fumonisin contamination is still controversial. Our main goal was to evaluate whether the higher carotenoid content in HC maize could affect its susceptibility to fungal infection and mycotoxin contamination. Therefore, the impact of N fertilization on fumonisin levels was not considered, despite other authors having reported a possible relation. In our experiment, the potential role of N fertilization in fumonisin content was offset by random sampling, i.e. all the plots were randomly sampled to obtain a representative sample, and the samples from each plot were combined to obtain an aggregate sample of each type of maize.

In maize fields located in Southern Europe, the fungal growth and mycotoxin production depend on the climatic conditions during the maize flowering period, as well as on the insect damage produced by *Ostrinia nubilalis* (European corn borer, ECB) and *Sesamia nonagrioides* (Mediterranean corn borer). The prevalence of *Fusarium* in our area is closely linked to the activity of these insects (Marín *et al.*, 2012). Injuries caused by insect pests allow the colonization of maize grains by fungi, but insects can also act as vectors (Munkvold and Desjardins, 1997). Thus, a reduction in insect pests can result in a lower frequency and/or lower severity of fungal infections and, consequently, a lower mycotoxin contamination, as shown by the comparison of fumonisin concentrations in Bt and non-Bt maize hybrids in different field locations (for further information see Annex III) (Díaz-Gómez *et al.*, 2016).

As part of this multidisciplinary project, other researchers from our university evaluated whether the higher carotenoid content in HC maize could affect the development (e.g. larval mortality, leaf consumption and larval weight) of two maize pests: ECB, which penetrates the stalks and cobs but also feeds on leaves, and armyworm moth (*Mythimna unipuncta*), which feeds mainly on the leaves. Similar results were obtained for both species, without significant differences in mortality rates after 14 days feeding on the leaves. However, both species consumed fewer leaves from HC maize

compared to leaves from its near isogenic line, but larval weight was not significantly affected. It should be noted that there was no difference in carotenoid content among leaves as transgenes are expressed under the control of endosperm-specific promoters. Nevertheless, HC maize leaves were less pigmented than M37W leaves, maybe due to a knock-on effect of carotenoid metabolism in the seeds, which affects the leaves and, consequently, how attractive they are to insect pests (Zanga, 2017). These results are supported by another experiment which showed that *S. nonagrioides* found Carolight® (an improved version of HC maize, for further information see page 20) maize plants less attractive as a place to lay eggs than near isogenic control plants (Cruz and Eizaguirre, 2015). Therefore, taking into account the difference in fumonisin levels between HC maize and its near isogenic line found in our experiments, and the results obtained by those researchers, it can be established that the high carotenoid content in HC maize grains can indirectly decrease the amount of damage caused by insects and thus minimize the opportunity for fungal infection and/or can reduce fumonisin biosynthesis or accumulation in maize plants. It is interesting to note that genetic engineering techniques can directly address mycotoxin biosynthesis, as recently shown by an aflatoxin-free transgenic maize in which aflatoxin biosynthesis has been silenced (Thakare *et al.*, 2017).

Thanks to the field experiments, HC maize and its near isogenic line could be used as raw material in poultry diets. Thus, mycotoxin levels were also determined in feed, but using enzyme immunoassay kits. Mycotoxin analysis in feed corroborated the results previously found in maize: control diets (based on M37W maize) had higher fumonisin levels than HC diets (based on HC maize) (for further information see Annex I). In spite of this, mycotoxin levels were below the maximum levels and the guidance values set for poultry feed by the European legislation (Table 1).

Table 1. Maximum levels for aflatoxin B_1 (AFB_1) and guidance values for other mycotoxins in poultry feed (in mg/kg relative to a feedingstuff with a moisture content of 12%) established by the European Union.

Mycotoxin	Level (mg/kg)	Source
AFB_1	0.02	European Commission, 2003
Total aflatoxins	0.02	European Commission, 2003
Fumonisins (B_1 + B_2)	20	European Commission, 2006
Deoxynivalenol	5	European Commission, 2006
Zearalenone	2–3	European Commission, 2006
Ochratoxin A	0.1	European Commission, 2006
T-2/HT-2 toxins	0.25	European Commission, 2013

2. High-carotenoid maize as feed ingredient

Maize is an important cereal source in poultry nutrition and thus a major source of energy (Leeson and Summers, 1997). Changes in the nutrient profile of the dietary ingredients used in poultry feed (e.g. an increase in the carotenoid content in maize grains) make it necessary to evaluate their effects on growth performance of broiler chickens for meat production and thus assess meat quality, including sensory evaluation (ILSI, 2007). In these evaluations, the choice of the comparator for genetically modified (GM) feed, which can be found in the near isogenic line (i.e. the parental line) is fundamental, but the inclusion of commercial varieties is also recommended to demonstrate the biological range of all measurements (EFSA, 2008). Comparison between the HC maize and its near isogenic line shows the benefit of the added nutrient (i.e. carotenoids), while comparison between HC maize and a commercial line provides further comparisons between nutritionally enhanced crops and commercial varieties.

Therefore, HC maize was used as feed component in two animal feeding trials to evaluate its effect on broiler productivity, health parameters and color evolution compared to diets based on its near isogenic line and a commercial standard yellow maize supplemented with natural color additives (Chapter II). The color differences among the types of diets evaluated in these feeding trials can be observed in Figure 4.

Figure 4. Color differences among types of diets used in the animal feeding trials reported in Chapter II. From left to right: control diet (based on M37W maize), high-carotenoid diet (based on HC maize) and commercial diet (based on standard yellow maize) before and after adding natural color additives (marigold flowers and red paprika extracts).

In addition to performance and animal health, the nutritional assessment of feed derived from GM plants with an intended modification of the nutrient content should include livestock studies to determine the bioavailability of individual nutrients in the GM feed (EFSA, 2011; ILSI, 2007). When it is necessary to assess the conversion of the precursors into the nutrients (e.g. β-carotene), at least the nutritionally enhanced crop and its near isogenic line supplemented with the nutrient under study should be included (EFSA, 2008). Generally, a bioavailability study must demonstrate that the target nutrient is efficiently digested and assimilated and then, once absorbed, has a positive effect (Carbonell-Capella *et al.*, 2014). The most appropriate animal model to study carotenoid metabolism should include the following characteristics: 1) absorb carotenoids intact at physiological levels, similar to humans; 2) have carotenoid distribution in serum and tissues similar to that of humans; 3) be representative of the disease of interest; 4) be readily available; 5) be easily manageable under laboratory conditions; and 6) be affordable (Lee *et al.*, 1999). Despite the fact that there is not a model that meets all of these criteria, chickens fulfill most of them as vitamin A and carotenoid metabolism in chickens is similar to that of humans (NRC, 1994; Pretorius and Schönfeldt, 2013). Therefore, the bioavailability of carotenoids supplied as intrinsic components of HC maize was evaluated in chickens in comparison to the same carotenoids provided by its near isogenic line supplemented with synthetic or natural color additives (Chapter III). This comparison shows the efficiency of HC maize.

Globally, HC maize did not adversely affect chicken growth and development, with similar values of body weight, feed intake and feed conversion ratio (FCR) to chickens fed on the other diets. Interestingly, the lowest FCR (i.e. kg feed per kg gain) was found in diets based on HC maize and its near isogenic line (M37W maize), resulting in better efficiency, which may be related to the genetic background of this maize line, although a diet based on M37W maize was the least efficient in a laying hen feeding trial (Moreno *et al.*, 2016). In our animal feeding trials, 24 animals per treatment were used, except for the bioavailability assay in which 8 animals per treatment were used (the minimum necessary to have statistical power). A high number of chickens is recommended to evaluate GM crops, usually 10–12 pens per treatment with 9–12 birds per pen (EFSA, 2008). Nevertheless, our studies were mainly limited by the production of the experimental field, which provided raw material to perform several experiments by different research groups, making it necessary to adapt the number of chickens to the kg of maize available each harvest.

In general terms, biochemical and hematological parameters did not substantially differ in chickens fed on the HC diet compared to those fed on the other diets, except for bilirubin levels which were higher in chickens fed on carotenoid-enhanced diets. This difference did not exist in a previous experiment in which chickens fed on the control and HC diets had total bilirubin values of 0.05 ± 0.003 and 0.06 ± 0.003 mg/dL, respectively (Nogareda *et al.*, 2016). A possible explanation could be a subclinical inflammation counteracted by an increase in bilirubin, which has immunomodulatory effects (Liu *et al.*, 2008). Furthermore, HC maize has previously been shown to promote poultry health and immunity: a heavier bursa of Fabricius was found in chickens fed on the HC diet than in those fed on the control diet, reflecting a possible better immunomodulatory response to vaccination against infectious bursal disease; a reduction in the severity of coccidiosis, an important poultry disease caused by protozoan parasites of the genus *Eimeria*, was found in chickens fed on the HC compared to those fed on the control diet when animals were experimentally challenged with 3 x 10^4 *E. tenella* oocysts orally; and lower incidences of footpad dermatitis and digital ulcers were found in chickens fed on the HC diet than in those fed on the control diet, in the presence and also in the absence of coccidiosis (Nogareda *et al.*, 2016).

Yellow skin is an abundant phenotype among domestic chickens, but its expression is influenced by the amount of carotenoids in the feed (Eriksson *et al.*, 2008). Like most other animals, chickens cannot synthetize carotenoids *de novo* and must obtain them from their diet (Breithaupt, 2007). Carotenoids supplied as intrinsic components of HC maize are bioavailable and confer to chickens a long-lasting golden pigmentation similar to animals fed on a commercial diet supplemented with natural color additives, although the latter provided the highest yellowness scores in breast and thigh muscles due to the marigold extract included in its composition (Breithaupt, 2007). Yellow and red xanthophylls were added to the commercial diet at similar levels to those commonly used in the poultry industry, approximately 10–fold more yellow than red xanthophylls (Amaya *et al.*, 2014). However, this ratio was adapted in the bioavailability assay to increase the concentration of provitamin A (PVA) carotenoids; thus, approximately 3–fold more red than yellow xanthophylls were added and, subsequently, the yellow scores were not as important. Xanthophylls supplied within the kernel matrix could replace external xanthophylls usually added to the poultry feed, as shown by HC maize.

The distribution of carotenoids in chickens is mainly dependent on the feed profile. Zeaxanthin, β-carotene, β-cryptoxanthin and violaxanthin are found at higher levels in the HC diet, whereas lutein is found at higher levels in the commercial diet supplemented with natural color additives. Accordingly, chickens fed on these diets accumulated these carotenoids in liver in similar proportions, except for β-carotene, due to its conversion into retinol. β-Carotene was not detected in the serum or liver of any chickens in any of the experiments, but the bioavailability study shows that β-carotene epoxides accumulate in the livers of chickens fed on the HC diet. In previous experiments, zeaxanthin and β-carotene epoxides were found in skin, fat and meat samples from chickens fed on the HC diet, suggesting that high levels of zeaxanthin and β-carotene could have accumulated initially, but then underwent oxidation (Nogareda *et al.*, 2016).

Nevertheless, nutrient bioavailability is a better indicator of nutritional quality than nutrient content alone (Sanahuja *et al.*, 2013). The levels of lutein, zeaxanthin and β-carotene from HC maize were bioavailable to the extent that they could be predicted based on the feed profile, whereas β-cryptoxanthin and violaxanthin from HC maize were found at higher levels in liver than those originally presented in the feed. The difference in nutrient bioavailability with respect to the feed composition was more pronounced in a laying hen feeding trial previously reported by our research group (Moreno *et al.*, 2016). In that trial, zeaxanthin and lutein concentration was 12.41 and 3.08 μg/g freeze-dried (fd), respectively, in the HC diet, whereas their concentration was 14.04 and 9.2 μg/g fd, respectively, in the livers of chickens fed on the HC diet.

PVA carotenoids (i.e. β-carotene and β-cryptoxanthin) from HC maize are bioavailable and contribute to liver retinol levels (Tables 2 and 3), although they are not metabolized in the same manner: β-carotene is preferentially converted into retinol in the intestine whereas β-cryptoxanthin is accumulated in the liver. This indicates that β-carotene is a better substrate for β-carotene 15,15'-diooxygenase 1 (BCDO1) than β-cryptoxanthin (Kim and Oh, 2009, 2010). The bioavailability assay showed that zeaxanthin may interfere with β-carotene absorption, as higher retinol levels were found in chickens fed on low zeaxanthin diets. Despite the fact that zeaxanthin is the most abundant carotenoid in HC maize, this biofortified maize provided bioavailable PVA carotenoids.

Table 2. Summary of results reported in Chapter II. Provitamin A (PVA) carotenoids and retinol levels in the three diets: control, high-carotenoid, and commercial (plus natural color additives) diets; serum and livers from animals reared on these diets. Values are means ± standard errors. For feed, n = 3; for serum, n = 6; for liver, n = 8. nd, not detected; ne, not evaluated; fd, freeze-dried.

Diet	PVA and retinol	Feed (μg/g fd)	Serum (μg/g mL)	Liver (μg/g fd)
Control diet	β-carotene	nd	nd	nd
	β-cryptoxanthin	nd	nd	nd
	Total PVA	nd	nd	nd
	Retinol	ne	2.95 ± 0.46	958.10 ± 83.78
High-carotenoid diet	β-carotene	3.06 ± 0.17	nd	nd
	β-cryptoxanthin	1.04 ± 0.06	nd	7.42 ± 0.51
	Total PVA	4.1	nd	7.42
	Retinol	ne	3.53 ± 0.38	1284.61 ± 78.97
Commercial diet + natural color additives	β-carotene	1.67 ± 0.00	nd	nd
	β-cryptoxanthin	0.84 ± 0.02	nd	0.26 ± 0.05
	Total PVA	2.51	nd	0.26
	Retinol	ne	3.81 ± 0.52	1001.01 ± 87.13

However, it must be borne in mind that vitamin A was added in all the diets (3 and 2.4 mg retinol/kg in starter and grower diets, respectively) to ensure normal growth and development, as its deficiency can cause a decrease in daily feed consumption, which would have prevented us from studying the use of HC maize as feed component. Inadequate vitamin A intake reduces the immune system response that contributes to disease susceptibility, whose symptoms are expressed to a larger extent in growing birds than in adults (NRC, 1994). An ideal bioavailability study to assess the conversion of PVA carotenoids to vitamin A would include the HC maize (without external supplementation) and its near isogenic line supplemented with the nutrient under study (EFSA, 2008). Nevertheless, it was decided to include vitamin A in both diets at the levels commonly used in the poultry industry (2.4 mg retinol/kg), as there was not enough maize to repeat the experiment if the animals did not grow correctly. Regardless of the vitamin A supplementation, there were differences among treatments (Tables 2 and 3), which must be mainly related to the additional PVA carotenoids provided by HC maize or by color additives.

Table 3. Summary of results reported in Chapter III. Provitamin A (PVA) carotenoid levels and retinol in the four diets: high-carotenoid diet, control diet supplemented with synthetic color additives (zeaxanthin, lutein and β-carotene), control diet supplemented with synthetic color additives (lutein and β-carotene but not zeaxanthin), control diet supplemented with natural color additives (marigold flowers and red paprika extracts); serum and livers from animals reared on these diets. Values are means ± standard errors. For feed, n = 3; for serum and liver, n = 4. nd, not detected; ne, not evaluated; fd, freeze-dried.

Diet	PVA and retinol	Feed (μg/g fd)	Serum (μg/g mL)	Liver (μg/g fd)
High-carotenoid diet	β-carotene	3.06 ± 0.17	nd	nd
	β-cryptoxanthin	1.04 ± 0.05	0.16 ± 0.02	5.69 ± 1.21
	Total PVA	4.1	0.16	5.69
	Retinol	ne	6.02 ± 0.80	727.50 ± 44.33
Control diet + zeaxanthin + lutein + β-carotene	β-carotene	2.72 ± 0.20	nd	nd
	β-cryptoxanthin	nd	nd	nd
	Total PVA	2.72	nd	nd
	Retinol	ne	5.85 ± 0.68	659.75 ± 73.21
Control diet + lutein + β-carotene	β-carotene	2.41 ± 0.11	nd	nd
	β-cryptoxanthin	nd	nd	nd
	Total PVA	2.41	nd	nd
	Retinol	ne	5.17 ± 0.25	902.23 ± 72.31
Control diet + natural color additives	β-carotene	2.24 ± 0.08	nd	nd
	β-cryptoxanthin	0.91 ± 0.05	nd	nd
	Total PVA	3.15	nd	nd
	Retinol	ne	5.14 ± 0.46	708.28 ± 85.13

The contribution of PVA carotenoids from HC maize to retinol liver levels is supported by the laying hen feeding trial in which external vitamin A was only added to the commercial diet (Moreno *et al.*, 2016). The retinol levels in the livers of hens fed on the control (based on the M37W maize), commercial (based on a commercial maize plus a 3 mg retinol per kg feed supplement), HC, and BKT (based on a biofortified maize rich in ketocarotenoids) diets were 380, 1454, 1397 and 1790 μg/g fd, respectively. Given that retinol supplement was not used in the biofortified maize diets

supplied to laying hens, the source of retinol must be the PVA carotenoids in the feed (Moreno *et al.*, 2016).

Vitamin A equivalence ratio is defined as the amount of dietary β-carotene that is equivalent to a unit of retinol and is measured using retinol activity equivalents (RAE) (Giuliano, 2017). The RAE for a mixed diet are 12:1 and 24:1 for β-carotene and other PVA carotenoids, respectively, and 2:1 for β-carotene in oil. Using the RAE, the vitamin activity of PVA carotenoids is half the vitamin A activity assumed when using the retinol equivalents (RE) (Institute of Medicine, 2001; NRC, 1989). Studies in Mongolian gerbils fed on conventional biofortified maize found that the β-carotene to retinol conversion ratio was 2.1–3.3:1 whereas the β-cryptoxanthin to retinol conversion ratio was 2.74:1 (Davis *et al.*, 2008a, 2008b; Howe and Tanumihardjo, 2006). The β-carotene to retinol conversion ratio was found to be 3.2–6.5:1 in studies involving humans (Li *et al.*, 2010; Muzhingi *et al.*, 2011). Generally, a vitamin A equivalence ratio of approximately 4:1 is applicable for biofortified cassava, yellow maize and Golden Rice, which is lower than ratios for vegetables that have more complex food matrices (10:1 to 28:1). Simple matrices have lower ratios and, consequently, higher conversion rates, than complex ones (Giuliano, 2017; Haskell, 2012; Van Loo-Bouwman *et al.*, 2014). Although the RAE recommended by the Institute of Medicine (2001) were applied in our bioavailability assay, it would be necessary to evaluate the retinol conversion ratio of PVA carotenoids from HC maize.

A further parameter to be considered in the evaluation of GM crops is the quality of food of animal origin (EFSA, 2008). The quality and sensory shelf life (Chapter IV) was evaluated in breast and leg quarter meat obtained from chickens fed on three different diets for 35 days: control, HC, and commercial (plus color additives) diets. It must be noted that it was not possible to test quality and sensory attributes related to meat consumption because HC maize is not yet approved for use as feed component. Thus, all the analyses were carried out using raw chicken meat. The design of the sensorial evaluation was adapted to the number of panelists, the professional panel (six assessors from the Food Technology and Animal Science Departments of the University of Lleida) evaluated meat samples stored directly under refrigerated conditions, while the consumer panel (a total of 237 people) evaluated meat samples frozen, thawed and stored under refrigerated conditions. This latter reversed storage design, which consists of having samples with different storage time all available on the same day,

allowed us to call consumers only on one day instead of during two weeks, which could have caused some bias. However, it was necessary to evaluate whether freezing and thawing processes had some effect on the quality measurements.

HC maize is suitable for use as feed component in poultry nutrition, providing meat with a good quality and shelf life as well as a long-lasting golden pigmentation. Breast and leg quarter meat obtained from chickens fed on the HC diet did not substantially differ from meat obtained from those fed on the other two diets in any of the criteria evaluated, except for color parameters, which were similar among chickens fed on carotenoid-enhanced diets. Regardless of the diet, meat samples were equally accepted after 7–9 days of storage, which could be established as the maximum storage period for this chicken meat accepted by consumers. These results were supported by the analysis of spoilage bacteria (*Pseudomonas* spp. and total aerobic mesophilic bacteria), which increased progressively from 1 to 9 days, and physical parameters (pH, color and texture), which remained quite similar during storage. Thus, the storage under refrigerated and frozen conditions did not substantially affect those parameters. The pigmentation of chicken meat obtained from animals fed on the different diets can be observed in Figure 5.

Figure 5. Color pigmentation of breast (above) and leg quarter (below) meat obtained from chickens fed on control, high-carotenoid and commercial (plus color additives) diets (from left to right).

An increase in tenderness has been related to β-carotene supplementation in poultry diets in other studies. Feed supplemented with β-carotene at 15 μg/g modified initial juiciness, tenderness and tooth adhesion in broiler thigh meat (Ruiz *et al.*, 2001), whereas feed supplemented with β-carotene at 1.5 μg/g showed the lowest hardness and tooth adhesion scores (Carreras *et al.*, 2004). Although the texture attributes could not be studied through a sensory analysis in our experiments, the evaluation of the hardness using a texture analyzer did not show considerable differences among diet treatments.

Despite the fact that the lipid oxidation was not studied, it is thought that the antioxidant activity of some carotenoids can contribute to protecting meat products against oxidative deterioration and thus improve the meat quality and shelf life. The lipid oxidation of animal products seems to be more efficiently retarded by the dietary supplementation with antioxidants rather than the post-mortem addition of antioxidants (Simitzis and Deligeorgis, 2011). Poultry diets supplemented with xanthophylls or marigold extract were effective in reducing lipid oxidation (Gao *et al.*, 2013; Rajput *et al.*, 2014; Wang *et al.*, 2017), whereas poultry diets supplemented with β-carotene were not as effective (Carreras *et al.*, 2004; Ruiz *et al.*, 1999). Therefore, it would be interesting to study the lipid peroxidation and to perform a sensory analysis with meat processed under dry or moist cooking in further studies.

Animal models are necessary to study the degree to which nutrients provided at source by biofortified crops are absorbed and to demonstrate that these crops can improve micronutrient status (Bouis and Saltzman, 2017). In spite of the fact that our findings are limited to broiler feeding studies, which are considered a sensitive model for comparing the nutritional equivalence of conventional and GM feed (EFSA, 2008), other authors have already evaluated the efficacy of PVA biofortified crops in humans. On the one hand, β-carotene derived from Golden Rice was shown to be effectively converted to vitamin A in humans (Tang *et al.*, 2009). On the other hand, conventional PVA-biofortified maize demonstrated an improvement of visual function among children with marginal or deficient vitamin A status and an increase in total body vitamin A stores as effectively as supplementation (Gannon *et al.*, 2014; Palmer *et al.*, 2016). Further research is needed to show that HC maize can improve nutritional status in populations at risk of micronutrient deficiencies as well as to fully understand the health impact of consuming biofortified crops. In the near future, randomized controlled efficacy trials will be necessary to confirm directly in humans the results found in animals.

3. High-carotenoid maize as food ingredient

The Millennium Development Goals (MDGs) were a set of eight international development targets for the year 2015, whose main aim was to improve the standards of health, socioeconomic status and education in the world's poorest regions. The developing regions have almost reached the main goal, cutting by half the proportion of people who suffer from hunger by 2015. Thanks to promoting access to better nutrition, undernourished people have decreased from 23% in the nineties to 13% in 2015 (FAO *et al.*, 2015; UN, 2015). However, hidden hunger is not readily visible, globally affecting one in three people, and resulting in different types of malnutrition, including vitamin A deficiency (VAD) (FAO *et al.*, 2015). The prevalence of VAD among children aged 6–59 months in low-income and middle-income countries has decreased from 39% in 1991 to 29% in 2013. Despite the reduction, this prevalence remains high in South Asia (44%) and sub-Saharan Africa (48%) (Stevens *et al.*, 2015). Vitamin A supplementation is recommended in infants aged 6–11 months (30 mg RE) and children aged 12-59 months (60 mg RE at least twice a year) in areas where VAD is a public health problem (WHO, 2011). Supplementation and fortification programs have been widely adopted, but VAD still persists in South Asian and sub-Saharan African countries. Thus, other dietary interventions are necessary to combat vitamin A disorders in those countries, such as PVA biofortified staple crops (Table 4), a cost-effective strategy to improve the health status of poor populations who cannot afford a diverse diet (Meenakshi *et al.*, 2010). Taking into account the food matrix, the micronutrient retention after processing (50%) and the bioconversion ratio (12:1), the following target levels for PVA content have been set: 15 μg/g for wheat, rice, maize and cassava, 20 μg/g for pearl millet and 30 μg/g for beans and sweet potato (Bouis *et al.*, 2011). More than 20 million people in developing countries are now growing and consuming conventional biofortified crops, including those biofortified with PVA (i.e. cassava, maize and sweet potato) thanks to the HarvestPlus program (Bouis and Saltzman, 2017). Higher levels of PVA carotenoids can be achieved using genetic engineering techniques, which specifically target the carotenoid biosynthetic pathway, rather than conventional breeding techniques (Bai *et al.*, 2011). However, the crop performance can differ considerably between laboratory and field conditions; thus, novel crops have to be tested under different environments (Zanga *et al.*, 2016a). Regarding HC maize, xanthophylls content remained quite similar between laboratory and field conditions,

whereas β-carotene levels were significantly lower under field conditions (Table 5). Nevertheless, it should be noted that the data from the laboratory conditions represent the first-generation of HC maize, which has experienced changes during its early development under greenhouse conditions. Furthermore, it is likely that β-carotene has undergone oxidation processes, which are the main factors responsible for causing β-carotene degradation under ambient conditions, as shown by biofortified sorghum (Che *et al.*, 2016).

Table 4. Examples of provitamin A (PVA) biofortified crops obtained through conventional breeding and genetic engineering. β-CE, β-carotene equivalents = μg β-carotene + ½ (μg α-carotene + μg β-cryptoxanthin) (Institute of Medicine, 2001). It must be borne in mind that those examples are not at the same stage of development.

Biofortification techniques	Crop	PVA content (dw, dry weight)	Source
Conventional breeding	Cassava	15 μg/g	HarvestPlus, 2017
	Maize	15 μg/g	HarvestPlus, 2017
	Sweet potato	32 μg/g	HarvestPlus, 2017
	Pumpkin	209–658 μg/g	Ribeiro *et al.*, 2015
Genetic engineering	Maize	10 μg/g dw	Zanga *et al.*, 2016a
	Sorghum	12 μg/g dw	Che *et al.*, 2016
	Cassava	30 μg/g dw	Failla *et al.*, 2012
	Rice	31 μg/g dw	Paine *et al.*, 2005
	Potato	31 μg/g dw	Li *et al.*, 2012
	Banana	55 μg/g dw β-CE	Paul *et al.*, 2017
	Tomato	819 μg/g dw	Fraser *et al.*, 2007
	Soya bean	845 μg/g dw	Schmidt *et al.*, 2015

Table 5. Main carotenoids (μg/g dw – dry weight –) present in high-carotenoid (HC) maize under laboratory and field conditions.

Carotenoid	Laboratory (Zhu *et al.*, 2008)	Field (Zanga *et al.*, 2016a)
β-Carotene	57 μg/g dw	6 μg/g dw
β-Cryptoxanthin	6 μg/g dw	4 μg/g dw
Lutein	10 μg/g dw	9 μg/g dw
Zeaxanthin	25 μg/g dw	23 μg/g dw

In this Doctoral Thesis, it has been previously mentioned that HC maize is able to provide the recommended daily intake of PVA in 200 g of grain, but this quantity was calculated according to the results obtained under laboratory conditions. Considering the results obtained in the experimental field, HC maize is able to provide one third of the vitamin A recommended dietary allowance (RDA) for children in 200 g of grain. In spite of this difference, HC maize could still contribute to alleviating vitamin A disorders, especially in sub-Saharan African countries where white maize (which is practically devoid of carotenoids) is usually consumed. Notwithstanding the foregoing, the high levels of lutein and zeaxanthin in HC maize, which can contribute to maintaining visual health and quenching and scavenging reactive oxygen species thanks to their antioxidant activity, must be considered (Nwachukwu *et al.*, 2016).

Maize and maize-derived products, a common staple food for more than 200 million people worldwide, have to be processed before consumption, but different cooking techniques can be applied (Nuss and Tanumihardjo, 2010). In sub-Saharan African countries, rural populations usually cook maize into porridge (McCann, 2005). As part of the biofortified crop development, the micronutrients retention after processing has to be determined to be sure that enough levels of micronutrients remain in cooked food for target populations (Bouis and Saltzman, 2017). HC maize-based porridges have been shown not only to preserve the initial carotenoid content, but also to enhance it due to the increase in carotenoid extractability from the food matrix. Regardless of the type of porridge, HC maize-based porridges provided at least 40 μg/g dry weight (dw) of total carotenoids. The color differences between porridges based on M37W and HC maize can be observed in Figure 6.

Figure 6. Color differences between M37W (left) and high-carotenoid (HC) (right) maize-based porridges after cooking.

Nonetheless, it must be taken into account that the analysis of the total carotenoid content has not allowed us to infer the potential of HC maize-based porridges to meet the dietary recommendations for vitamin A. It is well known that processing can change the profile and functionality of carotenoids (Rodriguez-Amaya, 2016). Hence, it would be necessary to apply HPLC (high performance liquid chromatography) techniques to know the PVA content provided by HC maize-based porridges. A good retention of PVA carotenoids has been reported in porridges (≥ 70%) and non-alcoholic beverages made with conventional biofortified maize (≥ 79%) (Awobusuyi *et al.*, 2016; Li *et al.*, 2007). Other transgenic biofortified crops, such as cassava, sorghum and potato, have also demonstrated a good bioaccessibility of PVA carotenoids (Chitchumroonchokchai *et al.*, 2016; Failla *et al.*, 2012; Lipkie *et al.*, 2013). Thus, PVA carotenoids provided by HC maize are also expected to have good retention and bioaccessibility after cooking.

Bearing in mind the results obtained in the animal feeding trials, where β-cryptoxanthin accumulated in the liver at higher levels than those initially presented in the feed, β-cryptoxanthin levels are likely to be higher than those reported under field conditions. Furthermore, biofortification seems to prevent the loss of PVA carotenoids during cooking more efficiently than standard fortification, probably due to a food matrix effect (for further information see Annex II) (Díaz-Gómez *et al.*, 2017). Therefore, HC maize can contribute to improving the health status of populations who depend on maize as a staple crop, and could even help to alleviate VAD in populations at risk. Different factors can affect the nutrient bioaccessibility and bioavailability in nutritionally enhanced crops (Figure 7, for further information see Annex II); thus, more studies are necessary to confirm the health effects.

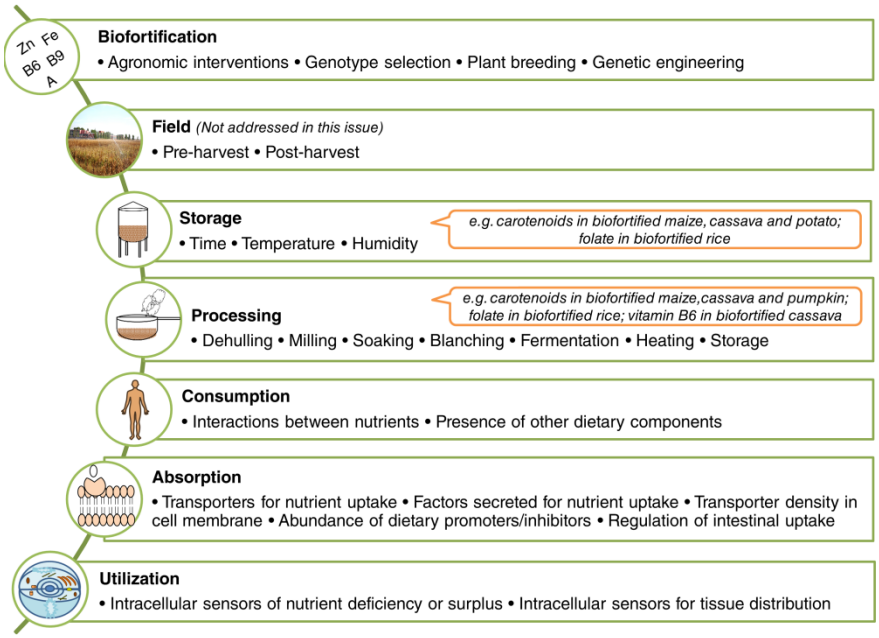

Figure 7. Main factors that affect the nutrient bioaccessibility and bioavailability in biofortified crops (Díaz-Gómez *et al.*, 2017).

Genetic engineering techniques are a useful tool to increase micronutrient levels as well as other desirable agronomic traits in crops. These techniques are the only option to enhance the content of nutrients which do not naturally exist at the required levels in the target crop (Bai *et al.*, 2011; Bouis and Saltzman, 2017). Nevertheless, the deployment of genetically engineered (GE) biofortified crops has to deal with several constraints mainly due to regulatory frameworks, as exemplified by Golden Rice, in which the economic costs of opposition in India have been calculated at approximately US$199 million per year for the last decade (Dubock, 2014; Wesseler and Zilberman, 2014). Nutritionally enhanced crops are not expected to be grown in the European Union (EU) and in developing countries economically dependent on the EU, as the legislative framework for the approval of GE crops, which is focused on the process rather than on the product, is the most restrictive worldwide (for further information see pages 32–33) (Masip *et al.*, 2013).

Policymakers need to understand that agriculture plays a fundamental role in health status improvement. Thus, their decisions must be based on available

scientific evidence rather than on beliefs (Moghissi *et al.*, 2016). Several independent scientific authorities in the world, including the United Nations Food and Agriculture Organization (FAO) and the European Commission, have concluded that GE crops are not more risky to humankind or the environment than crops derived from any other method of production (Dubock, 2014; European Commission, 2010; FAO, 2004). Recently, more than 100 Nobel Prize winners supported this claim, highlighting the importance of crops and foods improved through biotechnology, including PVA biofortified crops (Support Precision Agriculture, 2016).

Biofortified crops could address micronutrient deficiencies if they are adopted by farmers and consumed by target groups, which mainly depends on their cost-effectiveness (Meenakshi *et al.*, 2010). However, it must be noted that GE biofortified crops need to be previously approved for cultivation and then for human consumption. Education programs are necessary to promote the nutritional qualities of biofortified crops to both farmers and consumers. In fact, the adoption of biofortified crops by producers and distributors ultimately relies on consumer preferences, which are influenced by socioeconomic factors such as gender, education, income and ethnic background (Bett *et al.*, 2010; Muzhingi *et al.*, 2008). White maize is preferred by many African consumers due to the negative association of yellow maize with food aid and animal feed. Despite these cultural preferences, biofortified maize could make a difference thanks to its visual traits (i.e. deep yellow-orange kernels), which can easily be associated with its nutritional content (Birol *et al.*, 2015; Meenakshi *et al.*, 2012). Recently, orange biofortified maize has been shown not only to improve vitamin A status when consumed as a staple food, but also to be accepted by consumers (Tanumihardjo *et al.*, 2017). The HC maize technology package has already been transferred to the Council for Scientific and Industrial Research (CSIR), located in Pretoria (South Africa), and the Tamil Nadu Agricultural University (TNAU), located in Coimbatore (India). These scientific centers are expected to undertake the commercialization and distribution of the technology (Zanga *et al.*, 2016b). South Africa was the first African country to allow the commercial production of engineered crops as well as the first country worldwide to release a basic food staple, white Bt maize (Bett *et al.*, 2010). HC maize is likely to be well accepted by South African producers and consumers, and could therefore address preventable diseases, particularly those affecting children and women. Notwithstanding the foregoing, complex national and international rules are expected to postpone HC deployment.

This Doctoral Thesis evaluated whether HC maize could be suitable as feed and food ingredient. All the studies included here were developed in accordance with the European Food Safety Authority recommendations (EFSA, 2008, 2011) and other legislation (for further information see pages 32–34). HC maize and its derived food and feed were compared with their respective comparators (i.e. M37W maize and its derived food and feed), since traditionally cultivated crops have widely demonstrated their safe use for consumers and/or domesticated animals. The results presented contribute knowledge to the fight against food insecurity, especially in at-risk populations. Complementarily, the findings reported are an advance in the understanding of the carotenoid metabolism in animals. HC maize not only has the potential to improve human health in populations whose diet relies on cereals, but also its use as feed component can improve animal nutrition and, consequently, nutritious meat and other products will be obtained. The scientific articles obtained from this Doctoral Thesis will provide evidence for the future approval of HC maize.

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CONCLUSIONS

The main goal of this Doctoral Thesis was to ascertain whether high-carotenoid (HC) maize could be used as feed and food ingredient. Taking into account the results presented in this Doctoral Thesis, as well as the results found in other studies carried out in this university, we can conclude that HC maize is a cost-effective and sustainable source of carotenoids which can be used as feed and food component. Furthermore, HC maize may contribute to improving the health status of populations who depend on maize as a staple crop, and could even help to address vitamin A deficiency in at-risk populations. The conclusions drawn in relation to the objectives indicated at the beginning of this Doctoral Thesis are detailed below.

Objective I: Evaluate, for three consecutive harvest seasons, the susceptibility to fungal infection and fumonisin and aflatoxin contamination of HC maize and its near isogenic line in open field experiments.

- HC maize did not significantly differ from its near isogenic line (M37W maize) in fungal infection, although the percentage of infected grains was slightly lower in HC maize in 2 of the 3 years of study. Regarding fungal species involved in this infection, *Fusarium* was the dominant genus isolated in both types of maize in all the years of study, *F. verticillioides* being the most common species isolated within this genus. There was no fungal infection by *Aspergillus* spp. in any of the years of study and, consequently, there was no aflatoxin contamination.

- Fumonisin contamination was detected in both types of maize in all 3 years of study, but there were significant differences between maize types. HC maize had lower fumonisin levels compared to its near isogenic line, but this difference was only statistically significant in 2 of the 3 years of study. Fumonisin levels were below the maximum level allowed by European legislation for unprocessed maize, except for the M37W maize in the second harvest. Accordingly, meteorological conditions were related to a higher fumonisin contamination in both types of maize in the second harvest, which highlights the importance of climate in maize production, above all considering current climatic trends in the Mediterranean area.

- Bearing in mind the results found in HC maize, carotenoid content in maize grains may disrupt fumonisin biosynthesis or accumulation, which could be related to carotenoid antioxidant activity, as some carotenoids

can quench oxygen free radicals produced by plant cells as a defense response, contributing to reducing oxidative stress that modulates toxin biosynthesis.

Objective II: Evaluate whether HC maize could be a suitable replacement for color additives in poultry feed.

- HC maize can be used as feed component in poultry diets since none of the criteria evaluated in the animal feeding trials were adversely affected. Chickens fed on the HC maize diet grew normally and had similar productivity and health parameters to those fed on diets based on its near isogenic line and a commercial yellow maize (supplemented with color additives).

- HC maize has been demonstrated to be a suitable alternative to color additives since it provides sufficient levels of xanthophylls. Chickens fed on the HC maize diet developed similar pigmentation to those fed on a diet based on a commercial yellow maize supplemented with natural color additives commonly used in the poultry industry (i.e. marigold flowers and red paprika extracts). HC maize could also contribute to reducing the quantity of vitamin A added in the poultry diet as it provides carotenoids with vitamin A activity.

Objective III: Assess the bioavailability of provitamin A (PVA) carotenoids (β-carotene and β-cryptoxanthin) provided as intrinsic components of HC maize.

- PVA carotenoids supplied as intrinsic components of HC maize are bioavailable at least to the same extent as synthetic compounds and natural extracts. However, PVA carotenoids in HC maize are not metabolized in the same manner: β-carotene is preferentially converted into retinol in the intestine, whereas β-cryptoxanthin accumulates in the liver.

- The carotenoid levels found in feed, serum and liver support the need to evaluate the nutrient bioavailability rather than the total amount of nutrient provided. Lutein, zeaxanthin and β-carotene from HC maize were bioavailable to the extent that this can be predicted based on their levels in the HC feed, whereas β-cryptoxanthin and violaxanthin were

found at higher levels in the liver than originally presented in the feed, corroborating the results found in the other two animal feeding trials.

- Zeaxanthin seems to interfere with β-carotene absorption as higher retinol levels were found in chickens fed on diets with lower zeaxanthin levels. Despite the fact that zeaxanthin is the most abundant carotenoid in HC maize, retinol levels remained high in livers from chickens fed on the HC diet.

Objective IV: Investigate the effect of HC maize on chicken meat quality and shelf life.

- HC maize used as feed component in poultry diets delivers meat with a good quality and shelf life. Breast and leg quarters obtained from chickens fed on the HC diet were equally or better accepted by the consumer panel than meat obtained from those fed on diets based on its near isogenic line and a commercial yellow maize (supplemented with color additives). Specifically, the shelf life of chicken meat (frozen, thawed and stored under refrigerated conditions) from animals fed on the HC diet was approximately 8–10 and 5.5–8 days, for breast and leg quarter meat, respectively. In general, 7–9 days could be considered as the maximum storage period for this chicken meat accepted by consumers. These results were supported by the microbiological analyses. Spoilage bacteria (*Pseudomonas* spp. and total aerobic mesophilic bacteria) increased progressively from day 1 to 9 days in all chicken samples, reaching similar levels to those commonly found in poultry meat.

- In accordance with the animal feeding trials, meat obtained from animals fed on HC diet exhibited a similar pigmentation to meat obtained from those fed on a commercial diet supplemented with color additives. Chickens fed on carotenoid-enhanced diets exhibited a more intense red and yellow color than those fed on a diet based on the near isogenic line, whereas pH and firmness were not substantially different among treatments. All the physical parameters evaluated (pH, color and firmness) remained similar during storage; thus, refrigerated and frozen conditions did not affect meat quality.

Objective V: Ascertain the optimal conditions for preserving carotenoids in HC maize when it is cooked.

- HC maize not only can preserve its initial carotenoid content after thermal treatment, but also can enhance it thanks to the carotenoid extractability from the food matrix. The optimum thermal treatment for preserving carotenoid content in HC maize was 75 °C/10 min, followed by 75 °C/20 min and 75 °C/40 min. However, temperatures ≤ 95 °C combined with short cooking times (10–60 min) would also be acceptable, as carotenoid content only decreased when cooking was prolonged for 2 hours.

- Among the home-cooking techniques tested, the best technique for preserving carotenoid content in HC maize-based porridges is unfermented thin porridge. It would be useful to control pH levels in fermented products, as carotenoid levels in maize meals seem to be affected by low pH values. The preference for thin or thick porridges varies among regions, hence the need for nutritional programs to encourage the use of appropriate cooking techniques to preserve carotenoid content in biofortified staple crops at the same time as promoting their consumption.

FUTURE PROSPECTS

This Doctoral Thesis aimed to investigate whether high-carotenoid (HC) maize could be used as feed and food component. The findings support this hypothesis; thus, HC maize is a suitable ingredient for feed and food. As a result, this Doctoral Thesis includes seven scientific publications, specifically five research articles and two reviews. This research allowed us to answer some questions and to formulate new ones, but its main contribution could be its use in the legislative approval process for this genetically modified (GM) crop.

From my point of view, it would be interesting to evaluate more deeply the role of carotenoids in mycotoxin contamination, and their potential in the inhibition of fumonisin biosynthesis and/or accumulation, which makes it necessary to evaluate the role of each carotenoid individually. Bearing in mind the results found by other authors, the possible effect of the carotenoid content on insect feeding should be also considered.

Some results have a promising future, such as HC maize-based porridges, but it would be necessary to assess their direct consumption by rural populations to evaluate both carotenoid absorption and consumer acceptance. Accordingly, it would be essential to keep working on the bioavailability of provitamin A (PVA) carotenoids from HC maize meals, but it is fundamental to move from chicken models to humans. In this way, we will be able to make sure that PVA carotenoids from HC maize are absorbed and assimilated by target populations.

There are other interesting lines of research, such as the structural changes in carotenoids from HC maize after cooking and the role of these carotenoids in meat lipid peroxidation. However, the most important future prospects are related to the possible effects that the consumption of HC maize could have on the health status of rural populations. Clinical trials are necessary to ascertain whether HC maize can contribute to improving vitamin A status, without the need for fortification and/or supplementation.

As a conclusion, the use of HC maize as a staple food crop by at-risk populations in developing countries would be the icing on the cake, but we all know that it will be difficult to achieve this objective, at least with the current legislative framework. It is well-known that GM crops have some difficulties to be deployed, as exemplified by Golden Rice. We hope that the scientific centers in which HC maize seeds have already been released can overcome all the constraints and that one day we will see how HC maize helps to fight against food insecurity.

ANNEXES

ANNEX I

Mycotoxin levels in poultry feed

Mycotoxin levels in poultry feed

Mycotoxin levels in poultry feed were determined using enzyme immunoassay kits (Ridascreen®, R-Biopharm AG, Darmstadt, Germany) and the results obtained are shown in Tables 1 and 2. Mycotoxin analysis indicated that mycotoxins were below the maximum levels set for aflatoxin B_1 in poultry feed (0.02 mg/kg) and the guidance values set for other mycotoxins by the European Union (for further information see page 186).

Table 1. Mycotoxin analysis of the diets used in the first animal feeding trial. Values are means ± standard errors (n = 3). Means within a row with no superscript in common are significantly different ($p < 0.05$). LOD, limit of detection; nd, not detected; FBs, fumonisins; AFs, aflatoxins; AFB_1, aflatoxin B_1; OTA, ochratoxin A; ZEA, zearalenone; DON, deoxynivalenol; T-2/HT-2, T-2/HT-2 toxins. **a)** Starter diets (used on days 1–8); **b)** grower diets (used on days 9–35).

a)	Starter control	Starter high-carotenoid	Starter commercial
FBs (mg/kg)	1.21 ± 0.19 [a]	0.22 ± 0.01 [b]	0.43 ± 0.11 [b]
DON(µg/kg)	23.16 ± 2.78 [a]	18.15 ± 7.30 [a]	104.00 ± 16.00 [a]
T-2/HT-2 (µg/kg)	50.20 ± 12.23 [a]	61.7 ± 3.55 [a]	62.90 ± 11.05 [a]
OTA (µg/kg)	1.71 ± 0.11 [a]	1.65 ± 0.21 [a]	3.68 ± 1.52 [a]
ZEA (µg/kg)	nd	nd	nd
AFs (µg/kg)	< LOD	< LOD	< LOD
AFB_1 (µg/kg)	< LOD	< LOD	< LOD
b)	Grower control	Grower high-carotenoid	Grower commercial + color additives
FBs (mg/kg)	1.05 ± 0.15 [a]	0.52 ± 0.08 [b]	0.28 ± 0.04 [b]
DON(µg/kg)	30.90 ± 9.16 [a]	29.00 ± 6.22 [a]	106.67 ± 38.46 [a]
T-2/HT-2 (µg/kg)	54.00 ± 2.57 [a]	85.20 ± 15.40 [a]	49.73 ± 3.68 [a]
OTA (µg/kg)	2.13 ± 0.05 [a]	2.37 ± 0.16 [a]	2.37 ± 0.13 [a]
ZEA (µg/kg)	nd	nd	nd
AFs (µg/kg)	< LOD	< LOD	< LOD
AFB_1 (µg/kg)	< LOD	< LOD	< LOD

Table 2. Mycotoxin analysis of the diets used in the second animal feeding trial and in the bioavailability trial. Values are means ± standard errors (n = 3). Means within a row with no superscript in common are significantly different (p <0.05). LOD, limit of detection; FBs, fumonisins; AFs, aflatoxins; AFB_1: aflatoxin B_1; OTA, ochratoxin A; ZEA, zearalenone; DON, deoxynivalenol; T-2/HT-2, T-2/HT-2 toxins. **a)** Starter diets (used on days 1–8); **b)** grower diets (used on days 9–35).

a)	Starter control	Starter high-carotenoid	Starter commercial
FBs (mg/kg)	0.99 ± 0.02 [a]	0.22 ± 0.01 [c]	0.45 ± 0.03 [b]
DON(µg/kg)	< LOD	< LOD	88.00 ± 16.30
T-2/HT-2 (µg/kg)	< LOD	< LOD	< LOD
OTA (µg/kg)	< LOD	< LOD	< LOD
ZEA (µg/kg)	4.37 ± 0.68 [a]	5.77 ± 2.13 [a]	27.10 ± 18.26 [a]
AFs (µg/kg)	< LOD	< LOD	< LOD
AFB_1 (µg/kg)	< LOD	< LOD	< LOD
b)	Grower control	Grower high-carotenoid	Grower commercial + color additives
FBs (mg/kg)	0.32 ± 0.01 [b]	0.18 ± 0.01 [b]	0.52 ± 0.06 [a]
DON(µg/kg)	< LOD	< LOD	104.52 ± 7.16
T-2/HT-2 (µg/kg)	< LOD	< LOD	< LOD
OTA (µg/kg)	< LOD	< LOD	< LOD
ZEA (µg/kg)	5.16 ± 1.00 [b]	1.83 ± 0.34 [b]	17.67± 3.88 [a]
AFs (µg/kg)	< LOD	< LOD	< LOD
AFB_1 (µg/kg)	< LOD	< LOD	< LOD

ANNEX II

Biofortification of crops with nutrients: factors affecting utilization and storage

Joana Díaz-Gómez, Richard M. Twyman, Changfu Zhu, Gemma Farré, José C. E. Serrano, Manuel Portero-Otin, Pilar Muñoz, Gerhard Sandmann, Teresa Capell and Paul Christou

Current Opinion in Biotechnology (2017), 44: 1–9

Biofortification of crops with nutrients: factors affecting utilization and storage

Joana Díaz-Gómez [a], Richard M. Twyman [b], Changfu Zhu [c], Gemma Farré [c], José C. E. Serrano [d], Manuel Portero-Otin [d], Pilar Muñoz [c], Gerhard Sandmann [e], Teresa Capell [c] and Paul Christou [c, f]

[a] Department of Food Technology, University of Lleida – Agrotecnio Center, Lleida, Spain; [b] TRM Ltd., York, UK; [c] Department of Plant and Forestry Science, University of Lleida – Agrotecnio Center, Lleida, Spain; [d] Department of Experimental Medicine, University of Lleida – Biomedical Research Institute of Lleida (IRB Lleida), Lleida, Spain; [e] Biosynthesis group, Department of Molecular Biosciences, J. W. Goethe University, Frankfurt, Germany; [f] ICREA, Catalan Institute for Research and Advanced Studies, Barcelona, Spain

Abstract

Biofortification is an effective and economical method to improve the micronutrient content of crops, particularly staples that sustain human populations in developing countries. Whereas conventional fortification requires artificial additives, biofortification involves the synthesis or accumulation of nutrients by plants at source. Little is known about the relative merits of biofortification and artificial fortification in terms of nutrient bioaccessibility and bioavailability, and much depends on the biochemical nature of the nutrient, which can promote or delay uptake, and determine how efficiently different nutrients are transported through the blood, stored, and utilized. Data from the first plants biofortified with minerals and vitamins provide evidence that the way in which nutrients are presented can affect how they are processed and utilized in the human body. The latest studies on the effects of the food matrix, processing and storage on nutrient transfer from biofortified crops are reviewed, as well as current knowledge about nutrient absorption and utilization.

1. Introduction

Nutrients in the human diet ultimately come from plants, but all our major food crops lack certain essential micronutrients (vitamins and minerals) (Zhu *et al.*, 2007). The endosperm of cereal staples such as rice, wheat and maize are the most important source of calories for humans, providing ~23%,

~17% and ~10% of total global calories, respectively (Rawat *et al.*, 2013). However, endosperm tissue lacks sufficient amounts of vitamins (particularly vitamins A, E, C and folate) and minerals (particularly iron, zinc and selenium) (Gómez-Galera *et al.*, 2010; Zhu *et al.*, 2007). Iron and zinc deficiencies affect more than 50% of the human population, resulting in poor growth and development, an impaired immune system, fatigue, muscle wasting, sterility and even death (Gómez-Galera *et al.*, 2010; Rawat *et al.*, 2013). More than four million children worldwide suffer from severe vitamin A deficiency (VAD), including 250,000–500,000 per year who become partially or totally blind (Bai *et al.*, 2011). Women have a higher demand for vitamin A during pregnancy, and currently more than 20 million pregnant women in developing countries suffer from VAD (Bai *et al.*, 2011).

Strategies to address micronutrient deficiency include dietary diversification, nutritional supplements, fortification and biofortification (Gómez-Galera *et al.*, 2010; Rawat *et al.*, 2013; Zhu *et al.*, 2007). A combination of approaches is likely to provide the greatest overall benefit, but in some populations dietary diversification is impractical and supplements are only suitable as short-term interventions (Gómez-Galera *et al.*, 2010; Rawat *et al.*, 2013). Fortification requires the addition of nutrients to food products, for example, iodine is added to table salt, and iron, zinc and folate are added to flour to make bread (Gómez-Galera *et al.*, 2010; Rawat *et al.*, 2013). One major drawback of these approaches is the limited stability of the additives, for example, folate added to rice becomes more soluble at higher temperatures and is lost when the rice is boiled (Rawat *et al.*, 2013). A second disadvantage is that additives can also affect the quality of food, for example, iron additives are oxidized over time and this has an impact on taste (Gómez-Galera *et al.*, 2010). The third and major limitation of conventional fortification is that it is mainly suited to developed countries with the necessary technical infrastructure and distribution networks, but is less appropriate for developing countries with their extensive reliance on subsistence agriculture (Rawat *et al.*, 2013). Biofortification can address all three issues by facilitating the development of nutrient-dense staple crops that can be grown and distributed using existing agricultural practices (Gómez-Galera *et al.*, 2010; Saltzman *et al.*, 2013).

Biofortification is well established in principle but there are few practical examples of deployment thus far. Zinc-enriched rice and wheat have recently been deployed in Bangladesh and China, respectively; an orange sweet potato rich in provitamin A (PVA) carotenoids has been released in Mozambique and Uganda; and PVA rich maize has been released in Zambia and Nigeria (Saltzman *et al.*, 2013). Golden Rice II, the first transgenic biofortified crop

engineered with PVA carotenoids in the endosperm, has incurred multiple delays in terms of deployment. It is currently being backcrossed into locally adapted varieties in the Philippines, Indonesia, India and Bangladesh (Saltzman *et al.*, 2013). Multivitamin corn (registered as the protected variety Carolight® in Spain) was developed by transforming an elite white-endosperm South African inbred line with four genes representing three different vitamin biosynthesis pathways, increasing the levels of β-carotene, other carotenoids, vitamin C and folate (Naqvi *et al.*, 2009). Carolight® also contains a *Bacillus thuringiensis* (*Bt*) gene making it pest resistant (Zanga *et al.*, 2016). Biofortification is a sustainable approach which can bring nutritious staple crops to populations that are difficult to supply with supplements or fortified food products, and once the crop is developed there are no recurring costs other than those associated with normal agriculture. However, it is necessary to consider the efficiency of nutrient delivery by biofortified crops compared to other interventions in order to determine the long-term benefits of this approach. Data from the first biofortified crops are now available to allow such comparisons (Boxes 1 and 2).

Box 1. Glossary.

- *Supplementation* is the oral delivery of micronutrients in the form of pills, or powdered formulations that are dissolved before administration (Gómez-Galera *et al.*, 2010).
- *Fortification* is the practice of deliberately increasing the content of an essential micronutrient and thus improving the nutritional quality of food, for example, iron and zinc added to flour, and iodine added to table salt (Gómez-Galera *et al.*, 2010; WHO, 2016).
- *Biofortification* is the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology (WHO, 2016).
- *Bioaccessibility* is the amount of an ingested nutrient that is released from the food matrix in the gastrointestinal tract and becomes available for absorption (Carbonell-Capella *et al.*, 2014; Etcheverry *et al.*, 2012).
- *Bioavailability* is the amount of an ingested nutrient that is available for utilization or for storage, including gastrointestinal digestion, absorption, metabolism, tissue distribution, and bioactivity (Carbonell-Capella *et al.*, 2014; Etcheverry *et al.*, 2012).

Box 2. The measurement of bioaccessibility and bioavailability.

Nutrient bioaccessibility is usually determined *in vitro*, whereas nutrient bioavailability can be assessed either *in vitro* or *in vivo*. Solubility and dialysis assays measure bioaccessibility *in vitro* based on the proportional solubility of a nutrient in a test sample, and by equilibrium dialysis, respectively (Etcheverry *et al.*, 2012). They only measure the basic properties of nutrients and do not simulate the gut environment. Gastrointestinal models have been developed for the latter purpose and can simulate parameters such as saliva flow, peristalsis and intestinal pH (Cardoso *et al.*, 2015; Etcheverry *et al.*, 2012). Samples can be collected at any time during digestion, and bioavailability can be measured in addition to bioaccessibility when coupled to intestinal cells. However, such models usually do not incorporate homeostatic mechanisms, intestinal bacteria and hepatic metabolism (Carbonell-Capella *et al.*, 2014; Etcheverry *et al.*, 2012). Caco-2 cells are epithelial cells derived from a human colonic adenocarcinoma and they behave very much like intestinal cells when grown on plastic surfaces or Transwell inserts (Etcheverry *et al.*, 2012). Caco-2 cells form a polarized monolayer that accurately models the physical and biochemical barriers in the gut, thus providing a more realistic model of absorption including the secretion of chylomicrons, although certain aspects are missing such as other epithelial cell types, mucin and biofilms (Carbonell-Capella *et al.*, 2014; Etcheverry *et al.*, 2012).

Bioaccessibility and bioavailability can be investigated *in vivo* in humans or animal models, including complete balance studies (which estimate the bioaccessible or bioavailable fraction by comparing ingested and excreted quantities) and tissue analysis (including plasma/serum fractions). Humans are more appropriate for *in vivo* studies due to the well-documented differences in intestinal absorption mechanisms among rats, chickens and humans (Sanahuja *et al.*, 2013) although such methods are laborious and expensive and restricted by ethical constraints (Cardoso *et al.*, 2015).

2. Fate of nutrients produced in plants

The fate of organic nutrients in plant tissues is highly dependent on their solubility and their affinity for the constituents of the plant tissue matrix.

2.1. Folate

Folate is soluble in water and is easily released from the matrix, thus plasma folate levels are higher following the consumption of minced/chopped

spinach rather than whole leaves both as raw tissue (Castenmiller *et al.*, 2000) and after microwaving (van het Hof *et al.*, 1999). Dietary fibers such as cellulose, lignin, pectin and alginate do not appear to affect folate bioavailability (Ristow *et al.*, 1982). Baking causes the loss of endogenous bread folates (~40%) as well as added synthetic folic acid (30–60%). Furthermore, the bread matrix inhibits folate absorption (Öhrvik *et al.*, 2010).

2.2. Carotenoids

In contrast, the bioavailability of fat-soluble nutrients appears to be much more dependent on associations with matrix components and other dietary constituents, as shown for the six major dietary carotenoids (β-carotene, α-carotene and β-cryptoxanthin with PVA activity, lycopene, lutein and zeaxanthin without PVA activity) (Fernández-García *et al.*, 2012). Carotenoids are associated with proteins in many green leafy vegetables, whereas in carrots and tomatoes they are also stored in a semi-crystalline form (Ball, 2006; Hoffman and Gerber, 2015). Cooking, food processing, and the enzymatic processes during digestion weaken the cell walls and disrupt the protein-carotenoid complexes, promoting release and increasing bioavailability (Murador *et al.*, 2014). The bioavailability of carotenoids appears to depend on food particle size, with more efficient absorption from smaller food particles produced by homogenization, grinding, or milling. The bioavailability of carotenoids after release is favored by the presence of fats because carotenoids are incorporated into lipid droplets before entering the micelles, whereas soluble fibers, sterols and stanols inhibit the absorption of carotenoids (Goltz *et al.*, 2012; Hoffman and Gerber, 2015; Lakshminarayana and Baskaran, 2013; Yonekura and Nagao, 2007). The inhibitory effect of fibers may reflect the higher viscosity of fibrous solutions, the formation of gel aggregates, the incomplete hydrolysis of triacyl glycerols, or carotenoid aggregation (Yonekura and Nagao, 2009). Carotenoids are lipophilic and may also compete with plant sterols and stanols for solubilization in mixed micelles (Yonekura and Nagao, 2007).

2.3. Vitamin E

Vitamin E comprises eight fat-soluble molecules (α, β, γ and δ tocopherol and the corresponding tocotrienols, with α-tocopherol possessing the greatest biological activity) and like carotenoids its bioavailability is therefore highly dependent on interactions with the food matrix (DellaPenna and Mène-Saffrané, 2011; Nesaretnam, 2008). Accordingly, the bioaccessibility of vitamin E varies extensively in different types of food, ranging from 0.47%

in apple to almost 100% in banana, white bread and lettuce. Interestingly, the bioaccessibility of α-tocopherol was similar to that of γ-tocopherol when sourced from almonds, wheat germ, cheese or hazelnuts, but α-tocopherol was more bioaccessible than γ-tocopherol when sourced from banana, bread, lettuce and milk. This may reflect the food matrix effect, which determines the location of tocopherols, their physicochemical state, and the co-presentation of absorption effectors such as fibers, fats, sterols and stanols (Reboul *et al.*, 2006).

2.4. Calcium and iron

The bioavailability of minerals is affected by the food matrix, intrinsic chemical properties such as the oxidation state and counter-ion, and also by co-presented food substances, because all of these factors can affect solubility (Etcheverry *et al.*, 2012). Calcium must be solubilized before it can be absorbed. The extracellular calcium concentration depends on intestinal absorption, kidney reabsorption and bone resorption/formation, which are regulated by the calcium sensing receptor (CaSR) located in the parathyroid gland (Zhang *et al.*, 2015). The absorption of calcium is highly dependent on the abundance of phytate and oxalate, which can combine with calcium to form insoluble complexes (La Frano *et al.*, 2014; Liang *et al.*, 2010). Calcium also forms complexes with proteins, so cooking can help to release calcium for absorption, but the cooking method is important because the soluble calcium leaches into water used for boiling, but is retained during baking (Repo-Carrasco-Valencia *et al.*, 2010). Vitamin D is also required for calcium absorption (Heaney, 2008). Similarly, iron in meat and fish is relatively easy to absorb because of its favorable oxidation state and its storage in the form of ferritin-iron complexes that release the mineral readily, whereas some dietary proteins (such as albumin, casein, phosvitin and conglycinin) and certain plant polyphenols can reduce the bioavailability of iron (Collings *et al.*, 2013; Etcheverry *et al.*, 2012).

3. The role of the food matrix, food processing and storage

3.1. Food matrix

The major role of the food matrix in terms of nutrient bioaccessibility and bioavailability is to trap the nutrients within cells or subcellular compartments, and to provide constituents that interact chemically with specific nutrients to either encourage or delay their release, leading to their

classification as absorption promoters and inhibitors (Table 1). Lipid food components increase the bioaccessibility of fat-soluble nutrients, so cooking methods that preserve fats (e.g. frying) tend to outperform methods that disperse them (e.g. boiling) in terms of promoting the bioaccessibility of nutrients such as β-carotene, as recently shown for biofortified cassava (Berni *et al.*, 2014). Similarly, β-carotene bioaccessibility increased by threefold–fivefold in a transgenic biofortified sorghum line when the lipid content was increased from 5% to 10% (Lipkie *et al.*, 2013). Inhibitors such as phytate, oxalate and polyphenols reduce the bioaccessibility of iron and zinc by forming insoluble complexes. Transgenic maize, rice and sorghum with lower phytate levels in the seeds have been developed to address this issue (Raes *et al.*, 2014). Biofortification is advantageous for iron nutrition because plants can be engineered to maximize bioaccessibility. In contrast, standard fortification is achieved using sparingly soluble iron compounds to avoid an undesirable metallic taste, but the bioavailability of such compounds is low (Moretti *et al.*, 2014). Agronomic interventions are short-term strategies that focus on the use of soil and foliar mineral fertilizers, but regular applications are required (Carvalho and Vasconcelos, 2013). In maize, rice and wheat, foliar fertilization achieves higher levels of zinc accumulation than soil fertilization (Joy *et al.*, 2015). Mineral biofortification is most efficient when cereals are not consumed as flours, e.g. rice grain. Accordingly, zinc in rice grains biofortified using zinc-rich fertilizer is absorbed to a similar extent as the same rice variety fortified artificially with zinc immediately before consumption (Brnic *et al.*, 2016).

Table 1. Relationship between micronutrients and the food matrix.

Carotenoids (Ball, 2006; La Frano *et al.*, 2014)

- In photosynthetic plant tissues, carotenoids are bound to proteins in the inner chloroplast membrane, whereas in other tissues, such as fruits and endosperm, they are mainly found in chromoplasts. Carotenoids accumulate in the plastoglobuli of chloroplasts and chromoplasts, but in the latter they are also deposited as crystals.
- Cell walls, carotenoid-protein complexes and fibers trap carotenoids and inhibit absorption.
- The type and amount of fat can influence carotenoid absorption by promoting the excretion of bile salts, enhancing micelle formation and carotenoid solubilization.
- Xanthophylls are more hydrophilic than carotenes and are thus easier to absorb.

- Carotenoid *cis* isomers are more easily absorbed than *trans* isomers due to their greater polarity and solubility.

Iron and zinc (Gibson, 2007; Moretti *et al.*, 2014; Raes *et al.*, 2014; Suri and Tanumihardjo, 2016)

- Iron and zinc are chelated by other food components for transport (e.g. nicotianamine, peptides, proteins and organic acids). Proteins can also enhance absorption.
- Heme iron and organic zinc complexes are more readily absorbed than non-heme iron and inorganic zinc salts. The absorption of non-heme iron and zinc from plant-based foods can be enhanced by consuming meat, poultry, fish or seafood in the same meal.
- Phytate, oxalate, phenolic compounds and fibers form insoluble complexes with iron and zinc. The efficiency of complex formation depends on the chemical properties of the mineral, the pH and the presence of other compounds. Phytate binds preferentially to calcium and iron, limiting their bioavailability but increasing zinc bioavailability.
- Organic acids (such as ascorbate and citrate) and cysteine promote iron absorption. EDTA can promote iron and zinc absorption.
- Calcium can compete with iron for intestinal absorption; its effect on zinc has not been determined.

Water-soluble vitamins (Ball, 2006; Gibson, 2007)

- Vitamins that form complexes in the food matrix are absorbed less efficiently than free vitamins.
- Vitamins B1, B3, B6 and B9 can become trapped in the insoluble part of the food matrix in certain plant foods, reducing their bioaccessibility.
- Dietary fiber does not have a significant impact on the absorption of water-soluble vitamins.

3.2. Food processing

Food processing can enhance the bioaccessibility and bioavailability of nutrients by removing inhibitors or releasing nutrients from the food matrix (Table 2) but it can also reduce nutritional value. For example, most cereal grains are dehulled and milled before consumption, causing significant losses of minerals (Raes *et al.*, 2014) and certain vitamins (Dunn *et al.*, 2014). Carotenoid levels tend not to be affected by light milling, but greater losses are caused by heavy milling (Mugode *et al.*, 2014; Pillay *et al.*, 2014). Genetic engineering strategies that promote the accumulation of nutrients in the

endosperm rather than the bran or husk are therefore useful to increase the nutritional value of polished grains (De Steur *et al.*, 2015), as shown by the expression of enzymes that promote the synthesis of phytosiderophores in rice, leading to the modulation of endogenous metal transporter gene expression and the mobilization of zinc and iron from the bran to the endosperm (Banakar *et al.*, 2016). Cooking and thermal food processing methods such as pasteurization can destroy heat-sensitive organic nutrients such as folate and B6 group vitamins, and the cooking method can also encourage leaching, but the bioaccessibility of other nutrients can increase when they are released from the plant matrix by cooking. Transgenic biofortified cassava provides sufficient bioavailable vitamin B6 after cooking: the leaves and roots retain, respectively, ninefold and fourfold more non-phosphorylated B6 than non-transgenic cassava (Li *et al.*, 2015). Transgenic biofortified rice meets folate requirements even after cooking losses of 45% (100 g of rice contains 500 μg of folates) (Blancquaert *et al.*, 2015). Interestingly, only 43% of the original content of PVA carotenoids was retained in fortified rice grains after cooking, whereas iron, zinc, folic acid and vitamin B12 levels usually remained above 80% of the original value (Wieringa *et al.*, 2014). In contrast, β-carotene was retained when biofortified maize (Mugode *et al.*, 2014; Pillay *et al.*, 2014) and biofortified pumpkin (Ribeiro *et al.*, 2015) were cooked (> 72% and > 78%, respectively) suggesting that biofortification prevents the loss of PVA carotenoids during cooking more effectively than standard fortification, probably caused by the food matrix effect. In hens fed on transgenic biofortified maize, PVA carotenoids are preferentially diverted to the liver, whereas non-PVA carotenoids accumulate in the egg, in some cases doubling the initial concentration in the feed. When non-PVA carotenoids were supplied as intrinsic components of the transgenic biofortified maize, these nutrients were more efficiently absorbed than carotenoid additives in the standard commercial maize diet (Moreno *et al.*, 2016). Transgenic biofortified cassava also preserves the bioaccessibility of PVA carotenoids after processing, with a greater efficiency of β-carotene transfer to micelles (30–45%) than non-transgenic cassava (27–31%) (Failla *et al.*, 2012). In contrast, the transfer of β-carotene to micelles in transgenic sorghum was less efficient (1–5%) than in non-transgenic sorghum (6–11%) (Lipkie *et al.*, 2013). Several studies have highlighted the importance of genotype-specific effects on the retention of carotenoids during identical processing treatments, probably reflecting differences in the food matrix (Berni *et al.*, 2014; Failla *et al.*, 2012; Mugode *et al.*, 2014; Ribeiro *et al.*, 2015). The impact of cooking on the retention of β-

carotene also varies according to the genotype (Berni *et al.*, 2014; Mugode *et al.*, 2014; Ribeiro *et al.*, 2015), and genotype has a greater effect on the quantity of β-carotene in the micelle fractions than on the retention of β-carotene after processing (Failla *et al.*, 2012).

Table 2. Effect of processing on the micronutrient content of food.

Carotenoids (Ball, 2006; De Moura *et al.*, 2015; La Frano *et al.*, 2014)

Drying	Can reduce carotenoid levels but this depends on the drying method, the temperature/time combination and the genotype of the plant source.
Storage	Can cause the loss of carotenoids, but this depends on the crop species, genotype and storage conditions.
Milling	Increases carotenoid bioavailability because the food particle size is reduced.
Blanching	Enhances carotenoid retention because it inactivates peroxidases that can lead to the formation of undesirable colors and flavors, and carotenoids are released from carotenoid–protein complexes.
Fermentation	Does not usually affect carotenoid retention, and can also remove inhibitors and/or favor the accumulation of nutritional promoters.
Nixtamalization	Defined as soaking maize grains in an alkaline solution, which can reduce carotenoid levels. Bioaccessibility depends more on the subsequent processing/cooking method.
Heating	Can increase bioaccessibility by releasing carotenoids from plant tissues and disrupting carotenoid–protein complexes, although this depends on the plant source and the cooking method. Boiling and steaming retain more carotenoids than baking and frying. Exposure to light and long-term heating induces *trans*-to-*cis* isomerization resulting in the loss of PVA activity, although photoxidation is the main factor responsible of carotenoid isomerization.

Iron and zinc (Gibson, 2007; Pereira *et al.*, 2016; Raes *et al.*, 2014)

Dehulling	Reduces the level of inhibitors that prevent mineral uptake. This process removes the bran, reducing the amount of fiber and phenolic compounds, but phytate levels still remain high because phytate is also present in the germ. Significant quantities of minerals can also be lost, e.g. up to 50% of the iron in some grains, whereas zinc losses are more variable.
Milling	Degrades the cell wall, allowing minerals to interact with other components. Iron, zinc and phytate levels are reduced by milling, but the remaining iron and zinc is more bioavailable.
Soaking	Can reduce phytate levels by solubilizing phytate or activating endogenous phytases. However, blanching and soaking also cause the leaching of minerals.
Fermentation	Can degrade phytate through the action of microbial phytases. Fermentation can also enhance iron and zinc absorption because low-molecular-weight organic acids are produced during this process. The phytate content is reduced more during fermentation than during cooking.
Nixtamalization	Can reduce iron absorption by competing with calcium, although it may also improve zinc and iron absorption by reducing the phytate content.
Heating	Can enhance mineral absorption by softening the cell walls and removing inhibitors. Minerals are heat-stable, although losses can occur due to leaching. The bioaccessibility of iron is affected more than zinc by the cooking method.

Water-soluble vitamins (Ball, 2006; Gibson, 2007)

Drying	Can result in the loss of vitamins, especially air drying which promotes oxidation.
Storage	Does not appear to affect water-soluble vitamins, except B vitamins in long-term storage, and vitamin C due to oxidation during storage.
Dehulling Milling	The grain components are separated, resulting in significant losses of certain B vitamins that accumulate in the bran and germ.
Soaking	Reduces the levels of water-soluble vitamins by leaching.

Blanching	Inactivates enzymes that oxidize vitamin C but also encourages the loss of vitamin C by leaching.
Fermentation	Can increase the bioavailability of certain B vitamins (e.g. B2 and B3).
Nixtamalization	Can reduce the content of certain B vitamins (e.g. B1 and B3), but in some cases the remaining quantity can become more bioavailable (e.g. B3).
Cooking	Can release vitamins from the food matrix but can also destroy heat-sensitive vitamins (B1, B2 and C), although this depends on temperature/time combinations. The greatest losses during cooking occur due to vitamins leaching into the water, so steaming is preferred to boiling.

3.3. Storage

The stability of nutrients during storage is also an important consideration because biofortified maize loses a greater quantity of carotenoids during post-harvest storage than during cooking (Mugode *et al.*, 2014). As discussed above for cooking and processing, genotype has an important impact on carotenoid stability during storage (Ortiz *et al.*, 2016). Moreover, maize genotypes which lose more carotenoids during drying tend to lose less during storage (Suri and Tanumihardjo, 2016). Biofortified cassava was more susceptible to carotenoid losses during storage than white cassava with added red palm oil, suggesting that fatty acids can prevent carotenoid degradation (Bechoff *et al.*, 2015). Transgenic biofortified rice with improved folate stability was recently reported to maintain folate levels for 4 months when stored at 28 ºC (Blancquaert *et al.*, 2015). The sequestration of carotenoids in chromoplasts, which act as a metabolic sink, can enhance carotenoid levels during storage, as recently shown for transgenic potatoes (cv. Désirée) in cold storage for 5 months (Li *et al.*, 2012). Nevertheless, when the Phureja cultivar was used as the parental genotype (high carotenoid content in tubers) instead of Désirée (low carotenoid content in tubers), there were no significant changes in total carotenoid levels during cold storage (Campbell *et al.*, 2015).

4. Downstream behavior of absorbed nutrients

Nutrient supplements and fortified foods are provided in well-controlled doses to avoid toxicity. One concern about biofortification is that dosing would be more difficult to control, but recent studies have shown that the uptake of nutrients from biofortified crops is regulated at the level of

absorption from the gut, and also at the cellular level and by the modulation of storage reservoirs, based on the abundance of nutrients already in the body and the demand for certain nutrient molecules (Jeong and Guerinot, 2008).

Each vitamin and mineral has a specific transporter that facilitates its uptake from the gut, but some unrelated nutrients can also share the same transporter, as shown for the sodium-dependent multivitamin transporter that can mobilize pantothenic acid, biotin, α-lipoic acid and iodine (Quick and Shi, 2015). In this context, the transport of one nutrient can be inhibited in a concentration-dependent manner by other compounds that share the same transporter. Fat-soluble compounds are also mobilized by lipid transporters that vary in specificity. For example, carotenoids are absorbed via scavenger receptors (class B type 1 and Niemann-Pick type C1-like 1) that are selective for particular carotenoid molecules such as lutein (Sato *et al.*, 2012). Some nutrients can only be absorbed as a complex with a ligand that is secreted into the gut. For examples, intrinsic factor is secreted by gastric parietal cells to absorb cobalamin (vitamin B12), polyglutamyl folates must be processed by glutamate carboxypeptidase II, and the pancreatic secretion of γ-glutamyl hydrolase is necessary to release folate for absorption (Shafizadeh and Halsted, 2007).

The intestinal uptake of nutrients is adaptively regulated by the substrate level in the diet and depends primarily on the number of transporters in the apical and basolateral cell membranes of endothelial cells. For thiamin, this involves the transcriptional regulation of thiamin transporter-2 (Nabokina *et al.*, 2013). The production of nutrient-specific transporters is regulated at the level of transcription. High levels of nutrient bioavailability lead to the suppression of transcription and starvation causes the transporter gene to be induced. In some cases, specific epigenetic changes have been observed in the promoter of the transporter gene, for example, the oversupply of riboflavin leads to the epigenetic suppression of the riboflavin transporter gene (Subramanian *et al.*, 2015).

The transport of nutrients after absorption may also be regulated. For example, fat-soluble vitamins, carotenoids and ω-3 fatty acids (particularly docosahexaenoic and eicosapentaenoic acids) are transported in lipid vesicles that require chylomicron assembly and secretion, and these processes are inhibited when the corresponding nutrients are plentiful (Wang *et al.*, 2014). Similarly, iron is exported from enterocytes via ferroportin and its distribution is limited by the availability of transferrin. Finally, specific

intracellular sensors of nutrient bioavailability may regulate tissue distribution. For example, the absorption of iron is inhibited by the regulatory protein hepcidin which is stored in macrophages. Interestingly, hepcidin synthesis is sensitive to both circulating iron and intracellular iron stores because the macrophages communicate with hepatocytes to regulate hepcidin release via multiple indicator proteins, including transferrin and transferrin receptor-2 (Evstatiev and Gasche, 2012).

5. Conclusions

The biofortification of staple crops was envisaged as a sustainable strategy to deliver nutritious food to populations that are unsuitable for other intervention measures, but the bioavailability of nutrients in biofortified crops must be confirmed before they can be widely deployed. The bioavailability of nutrients is partly dependent on the intrinsic qualities of each nutrient molecule and partly dependent on their presentation in the context of the food matrix.

The major difference between biofortification and standard fortification is that the latter involves additives that are mixed with the food, whereas biofortification embeds the nutrients inside plant cells. The bioencapsulation of nutrients in this manner can prevent them from leaching during cooking and processing, as shown by the direct comparison of β-carotene levels after cooking fortified and transgenic biofortified rice, but can also enhance the binding of nutrients to plant proteins and fibers, as shown for iron and other minerals. The full value of biofortified crops can therefore be realized only by combining the adoption of biofortified varieties with the most appropriate food preparation and cooking methods to maximize the bioavailability of different nutrients. Moreover, cooking and storage losses could be reduced by growing crops in which the nutrients are more stable (e.g. transgenic folate-biofortified rice). Biofortified crops can help to alleviate micronutrient deficiency in at-risk populations in a sustainable manner. Some biofortified crops (e.g. rice, maize, cassava and pumpkin) achieve better results than others (e.g. sorghum), but rural populations are accustomed to eating staple crops commonly harvested in their area, so biofortification strategies must be tailored for different communities to achieve the greatest improvements in nutritional health.

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ANNEX III

The impact of *Bacillus thuringiensis* technology on the occurrence of fumonisins and other mycotoxins in maize

Joana Díaz-Gómez, Sonia Marín, Teresa Capell, Vicente Sanchis and Antonio J. Ramos

World Mycotoxin Journal (2016), 9 (3): 475–486

The impact of *Bacillus thuringiensis* technology on the occurrence of fumonisins and other mycotoxins in maize

Joana Díaz-Gómez [a], Sonia Marín [a], Teresa Capell [b], Vicente Sanchis [a], Antonio J. Ramos [a]

[a] Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, Av. Rovira Roure, 191, 25198, Lleida (Spain);
[b] Plant Production and Forestry Science Department, University of Lleida, Agrotecnio Center, Av. Rovira Roure, 191, 25198, Lleida (Spain)

Abstract

In many developing countries, maize is both a staple food crop and a widely-used animal feed. However, adventitious colonization or damage caused by insect pests allows fungi to penetrate the vegetative parts of the plant and the kernels, the latter resulting in mycotoxin contamination. Maize seeds contaminated with fumonisins and other mycotoxins pose a serious threat to both humans and livestock. However, numerous studies have reported a significant reduction in pest damage, disease symptoms and fumonisin levels in maize hybrids expressing the *Bacillus thuringiensis* (Bt) gene *cry1Ab*, particularly in areas where the European corn borer is prevalent. When other pests are also present, the *cry1Ab* gene alone offers insufficient protection, and combinations of insecticidal genes are required to reduce damage to plants caused by insects. The combination of Cry1Ab protein with other Cry proteins (such as Cry1F) or Vip proteins has reduced the incidence of pests and, indirectly, mycotoxin levels. Maize hybrids expressing multiple Bt genes, such as SmartStax®, are less susceptible to damage by insects, but mycotoxin levels are not routinely and consistently compared in these crops. Bt maize has a greater economic impact on *Fusarium* toxins than aflatoxins. The main factors that determine the effectiveness of Bt hybrids are the type of pest and the environmental conditions, but the different fungal infection pathways must also be considered. An alternative strategy to reduce mycotoxin levels in crops is the development of transgenic plants expressing genes that protect against fungal infection or reduce mycotoxin levels by *in situ* detoxification. In this review article, we summarize what is known about the relationship between the cultivation of Bt maize hybrids and contamination levels with different types of mycotoxins.

Keywords

Fumonisins, aflatoxins, deoxynivalenol, *Bacillus thuringiensis*, European corn borer

1. Introduction

Maize (*Zea mays* L.) is the most common source of fumonisins in human and animal diets. Maize seeds are often contaminated with fumonisins produced primarily by *Fusarium verticillioides* and *F. proliferatum*. These fungi can infect the seeds, or the silks can be contaminated by airborne or waterborne conidia, systemic infections can be caused by contamination of the roots, or pest insects can injure the plants allowing fungal penetration (Munkvold and Desjardins, 1997). Other mycotoxins may be present alone or together with fumonisins, including aflatoxins, deoxynivalenol (DON), zearalenone (ZEA), and some recently-discovered *Fusarium* metabolites collectively known as emergent mycotoxins, such as moniliformin (MON), beauvericin, enniatins and fusaproliferin (Desjardins, 2006; Marín *et al.,* 2013). Aflatoxins are mainly produced by *Aspergillus flavus*, which synthesizes type B aflatoxins as well as cyclopiazonic acid (CPA) depending on the strain, and by *A. parasiticus*, which synthesizes both type B and type G aflatoxins, but not CPA. DON and ZEA are mainly produced by *F. graminearum* and *F. culmorum* and they are often found as co-contaminants. Among the emergent mycotoxins, MON is the most prevalent and can be produced by several *Fusarium* species, including *F. avenaceum*, *F. tricinctum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (Marín *et al*., 2013).

The consumption of mycotoxin-contaminated kernels is associated with a range of diseases and disorders in humans and domestic animals, including cancer, immune system dysfunction and metabolic disorders (Marasas, 2001; Marín *et al*., 2013; Sobrova *et al*., 2010; Williams *et al*., 2004). Fumonisins and aflatoxins are carcinogens (AFB_1 is the most carcinogenic natural compound known), and this creates a strong impetus to restrict the exposure of human and animal populations as far as possible (IARC, 1993, 2002, 2012). In 2001, several countries submitted information on the concentration of fumonisins in maize and maize-derived foods, and fumonisins were detected in more than 60% of all food products (JECFA, 2001). These data are supported by a European Union (EU) report on the exposure of the EU population to *Fusarium* toxins. Among samples of raw maize material, 67% were positive for FB_1 (total = 801) and a 51% were positive for FB_2 (total = 544) (SCOOP, 2003).

The European Commission (EC) and European Parliament (EP) have set maximum levels for mycotoxins in maize and maize products. When such products are intended for human consumption, these values are currently 200–1000 μg/kg for fumonisins, 750 μg/kg for DON, 100 μg/kg for ZEA, 3 μg/kg for ochratoxin A (OTA), 5 μg/kg for AFB_1, and 10 μg/kg for total aflatoxins. Recently, indicative levels of 100 μg/kg in maize were also established for the total content of the trichothecene mycotoxins T-2 and HT-2 (EC, 2006b, 2007, 2010, 2012, 2013a). In animal feed, only the level of AFB_1 is currently regulated in the EU (maximum 0.005–0.02 mg/kg, depending on the type of feed). Other important mycotoxins in feed are limited by guidance values that vary with the species of livestock: 0.9–12 mg/kg for DON, 0.1–3.0 mg/kg for ZEA, 0.05–0.25 mg/kg for OTA and 5–60 mg/kg for fumonisins (FB_1 + FB_2). Recently, indicative levels of 250–2000 μg/kg in cereal products for feed and compound feed have been specified for the total content of T-2 and HT-2, with the exception of feed for cats, for which the guidance value is 50 μg/kg (EC, 2002, 2003b, 2006a, 2013a,b).

In contrast, the United States Food and Drug Administration (FDA) has proposed a guideline for total fumonisin levels in food of 2–4 mg/kg (depending on the product), and total aflatoxin levels of 20 μg/kg in maize and maize products for human consumption. For animal feed, the levels vary from 5 to 100 mg/kg for fumonisins and from 20 to 300 μg/kg for aflatoxins, depending on the animal species (FDA, 2000, 2001).

2. Transgenic maize and mycotoxins

2.1. Factors that affect mycotoxin occurrence

The presence of mycotoxins in maize results from the interaction of several factors, including temperature and humidity, nutrient availability, the presence of other fungi, stress, and physical damage caused by pest insects. Before harvest, important factors include the weather (temperature, humidity and rainfall), exposure to insect pests, fungi and other pathogens, planting dates, the maize genotype and cropping system. Fumonisin contamination in maize is directly associated with *Fusarium* pink ear rot (mainly produced by *F. verticilliodes*) and its incidence depends on both environmental conditions and pest damage. Kernel damage caused by insects exposes the kernels to fungal spores, although there are several additional infection pathways. Fungal growth and mycotoxin accumulation can also be stimulated post-harvest by

poor storage conditions such as high humidity and the presence of other pests (Marín *et al.*, 2004; Miller, 2001).

Fungal growth and mycotoxin production are affected by multiple ecophysiological factors. The main factors that control fumonisin production in grain are temperature and water activity (a_w). *F. verticillioides* and *F. proliferatum* germinate at 5–37 ºC when a_w exceeds 0.88, although the growth range fluctuates in the range 7–37 ºC when a_w exceeds 0.90. The optimum conditions for fumonisin production by *F. verticillioides* are 30 ºC at 0.97 a_w and for *F. proliferatum* the corresponding values are 15 ºC at 0.97 a_w. Physicochemical and nutritional factors such as pH and carbon/nitrogen ratio can also affect fumonisin production. The presence of other fungi, such as *A. flavus* and *A. niger*, can affect the growth of *Fusarium* species, which is most competitive at 15 ºC and 0.98 a_w. At high a_w values, fumonisin production can be stimulated by *A. niger* and other species (Marín *et al.*, 1999; Picot *et al.*, 2010; Sanchis *et al.*, 2006).

2.2. Suppression of insect pests using Bt technology

Injuries caused by insects are common sites of fungal infection on maize ears and stalks. The fungi can be airborne or may be suspended in water droplets that splash the wound, but insects can also act as vectors. One of the most prevalent examples is the European corn borer (ECB) (*Ostrinia nubilalis* Hübner), a maize pest that not only injures plants and exposes them to infection, but also vectors fungal spores, particularly *F. verticillioides* and *F. proliferatum*. ECB therefore promotes *Fusarium* infection of maize kernels and stalks, and may reduce yields by increasing the incidence of stalk rot (Munkvold and Desjardins, 1997; Munkvold *et al.*, 1997; Sobek and Munkvold, 1999). *F. verticillioides* is the most prevalent fungal pathogen of maize but fungicides are only partially effective, with efficacy depending on the pathogen strain and the fungicide mechanism of action (Falcão *et al.,* 2011). Therefore, pest insects are more appropriate targets than fungi for the development of strategies to reduce mycotoxin levels in maize.

In many parts of the world, the management of ECB now relies on transgenic hybrid maize lines expressing the *cry1Ab* gene from the Gram-positive soil bacterium *Bacillus thuringiensis* (Bt). This gene encodes a potent pro-toxin that is activated in the alkaline environment of the insect gut and is highly specific towards particular insect species. These insecticidal crystal proteins are also named δ-endotoxins or Cry proteins. *B. thuringiensis* has been used since 1938 to produce an insecticidal spray, but Bt transgenic plants resistant to ECB larvae were first made available in the USA in 1996

and in the EU in 1998. Different strains of the bacterium express different *cry* genes producing different pro-toxins that can protect plants against many different pests, including the corn rootworm complex (*Diabotrica virgifera*) (EPA, 2011; Höfte and Whiteley, 1989; Koziel *et al.*, 1993; Schnepf *et al.*, 1998). Therefore, alternative Bt genes (such as *cry1F*) have also been expressed in maize to protect against further lepidopteran pests (Abbas *et al.*, 2013; Bowers *et al.*, 2013; Koziel *et al.*, 1993). Economically-important maize pests that can be partially controlled using Bt hybrids include the corn earworm (CEW; *Helicoverpa zea*), common stalk borer (*Papiapema nebris*), southwestern corn borer (SWCB; *Diatraea grandiosella*) and western bean cutworm (WBC; *Striacosta albicosta*), whereas this strategy has proven less efficient against the fall armyworm (FAW; *Spodoptera frugiperda*) and black cutworm (*Agrotis ipsilon*) (Bowers *et al.*, 2013, 2014; Dowd, 2000; Munkvold and Hellmich, 1999; Williams *et al.*, 2002, 2005, 2006). Table 1 lists the Bt events targeting lepidopteran pests and the corn rootworm complex that are currently commercially available in the USA.

Table 1. Current Bt maize varieties against lepidopteran pests and corn rootworm complex (EPA, 2011).

Pest	Bt event	Protein(s) expressed	Target pests[1]	Registrant
Lepidopteran pests	Bt11	Cry1Ab	ECB	Syngenta
	Mon810	Cry1Ab	ECB	Monsanto
	TC1507	Cry1F	ECB, BCW, FAW, SWCB	Dow/ Mycogen Pionner/ Dupont
	Mon89034	Cry1A.105 + CryAb2	ECB, SWCB, CEW, FAW	Monsanto
	MIR162	Vip3Aa20	CEW, FAW, BCW, WBC	Syngenta
Coleopteran pests	DAS-59122-7	Cry34Ab1 + Cry35Ab1	WCRW, NCRW, MCRW	Pionner/ Dupont
	Mon88017	Cry3Bb1	WCRW, NCRW, MCRW	Monsanto
	MIR604	Cry3A	CRW	Syngenta

[1] ECB: European corn borer; CEW: corn earworm, WBC: western bean cutworm; BCW: black cutworm; FAW: fall armyworm; SWBC: southwestern corn borer; CRW: corn rootworm; WCRW: western corn rootworm; NCRW: northern corn rootworm; MCRW: Mexican corn rootworm.

Since its adoption in the USA, Bt maize has become the second most widely cultivated GM crop worldwide, after herbicide-tolerant soybean. About 30% of global maize production in 2014 (184 million hectares) was represented by GM varieties (55.2 million hectares) (James, 2014). However, the EU has a strict, complex and contradictory legislative framework for GM crops, with only the Mon810 maize event currently authorized for cultivation (EC, 1998, 2003a, 2008).

2.3. Fumonisin contamination in Bt and non-Bt maize

The ability of Bt genes to protect maize against ECB and other lepidopteran pests means that Bt maize tends to suffer a lower frequency of fungal infections and the infections that occur are often less severe or even symptomless. In the case of fumonisins, there is a large body of evidence to support the benefits of Bt maize, as shown by the comparison of fumonisin concentrations in Bt and non-Bt maize hybrids in different field locations (Supplementary Table S1).

Munkvold *et al.* (1999) published a fundamental study concerning the effect of Bt maize on disease management and concluded that transgenic hybrids expressing *cry1Ab* were less susceptible to ECB, suffered less from *Fusarium* ear rot and had lower fumonisin levels than their non-transgenic counterparts. However, if other insect pests were present alone or concurrent with ECB then the levels of fumonisins remained high (Dowd, 2000; Clements *et al.*, 2003; Hammond *et al.*, 2004; Papst *et al.*, 2005).

Many studies of natural infestations confirm the significant reduction in fumonisin levels associated with Bt hybrids (Abbas *et al.*, 2013; Ostry *et al.*, 2010; Pazzi *et al.*, 2006). These studies were carried out at different times in different countries, including Italy (Masoero *et al.*, 1999; Pietri and Piva, 2000), France (Bakan *et al.*, 2002; Folcher *et al.*, 2010; Pinson *et al.*, 2002), Spain (Bakan *et al.*, 2002), Argentina (Barros *et al.*, 2009; De la Campa *et al.*, 2005) and the USA (Abbas *et al.*, 2006, 2007, 2013; Bruns and Abbas, 2006; Dowd, 2001).

Importantly, Bowers *et al.* (2013) confirmed that lower levels of fumonisins were present in Bt hybrids exposed to ECB, but found that a *cry1Ab* x *vip3Aa* hybrid was more resistant to ECB, CEW and WBC than the *cry1Ab*

hybrid and the non-Bt hybrid in all of the years covered by the study. The vegetative insecticidal protein Vip3Aa can therefore be combined with other toxins such as Cry1Ab to target additional lepidopteran pests. Unlike the *cry* genes, which are expressed during sporulation, *vip* genes are expressed during the *B. thuringiensis* vegetative growth phase and they do not share sequence homology with *cry* genes (Lee *et al.*, 2003; Schnepf *et al.*, 1998). The *cry1Ab* x *vip3Aa* hybrid also showed a lower level of pest damage, a lower incidence of *Fusarium* ear rot and lower levels of fumonisins when infested with ECB, whereas *cry1F* hybrids were better protected against WCB because *cry1F* specifically targets this pest (Bowers *et al.*, 2014). A comparison of eight commercially available Bt hybrids expressing multiple genes found no significant differences in the content of fumonisins among the hybrids (Abbas *et al.*, 2013). Recent studies of SmartStax® maize, which produces Cry1F, Cry1A.105 + Cry2Ab2, Cry34Ab1/Cry35Ab1 and Cry3Bb1 to protect against common lepidopteran pests and the corn rootworm complex, reported less pest damage compared with single Bt hybrids and non-Bt hybrids, but did not consider mycotoxin levels (Rule *et al.*, 2014; Head *et al.*, 2014).

2.4. Aflatoxin contamination in Bt and non-Bt maize

Whereas the link between Bt maize and lower fumonisin levels is clearly established, the data for aflatoxin contamination are more contentious (Ostry *et al.*, 2014). Windham *et al.* (1999) showed a significant correlation between fungal and insect exposure, inoculation or infestation dates and aflatoxin contamination. They found that Bt hybrids suffered less damage from insects and had lower aflatoxin levels than the other hybrids studied (*A. flavus* resistant and *A. flavus* susceptible hybrids and a non-Bt isogenic hybrid) when manually infested with SWCB. More recent studies have focused on the inoculation technique, showing that inoculation with *A. flavus* by kernel wounding, which facilitates fungal penetration, results in high-level aflatoxin contamination regardless of the hybrids used. There were no differences among the hybrids when infected with fungi alone because lower aflatoxin levels in the Bt hybrids reflected the reduction in insect damage, which indirectly reduced fungal contamination. In contrast, a non-wounding inoculation technique combined with SWCB infestation resulted in significantly lower levels of aflatoxin in the Bt hybrids (Williams *et al.*, 2002, 2005; 2006). A testcross involving aflatoxin-resistant and aflatoxin-susceptible lines crossed with Bt and non-Bt maize revealed lower aflatoxin levels in the Bt testcrosses, but the difference for individual lines was

significant in only two of 10 lines investigated. The low insect pressure during the experiment could explain these results, because the higher the insect pressure, the greater the differences between hybrids (Williams *et al.*, 2010).

In a 3–year study, Wiatrak *et al.* (2005) observed significantly lower aflatoxin levels in Bt compared to non-Bt hybrids during the first year of the experiment, but there was no difference with a tropical non-Bt hybrid. In the second year, significantly lower levels of aflatoxins were detected in the Bt hybrids than the tropical non-Bt hybrid, but there were no differences compared to the non-Bt hybrids. In the final year, there were no differences in aflatoxin contamination among the hybrids. Another 3–year study reported lower levels of aflatoxins in Bt than non-Bt hybrids, but only in one of the years (Abbas *et al.*, 2006; 2007; Bruns and Abbas, 2006). Nevertheless, a subsequent study showed that aflatoxin contamination was significantly reduced in the Bt hybrid compared to its non-Bt isoline (Abbas *et al.*, 2008). The authors continued these field trials until 2009, reporting lower mycotoxin levels in Bt maize, but the difference was not significant, perhaps due to the continuous cultivation (Abbas *et al.*, 2013).

In the USA, Odvody *et al.* (2000) observed less insect damage in Bt hybrids but aflatoxin levels were not consistent. In a subsequent study of different Bt hybrids, the lowest level of insect damage was observed in the Mon840 event (*cry2Ab*) correlating with significantly lower aflatoxin levels compared to non-Bt and *cry1Ab* hybrids in 2000, but only compared to the non-Bt hybrid in 2001 (Odvody and Chilcutt, 2002). Different *cryAb* events were also evaluated, revealing less insect damage in the Bt hybrids Mon810 and Bt11 compared to non-Bt hybrids, but aflatoxin levels were also inconsistent in this experiment (Odvody and Chilcutt, 2003). Similarly, Maupin *et al.* (2001) did not find significant differences in the levels of ear rot and aflatoxin accumulation when comparing Bt and non-Bt hybrids inoculated with *A. flavus*. Buntin *et al.* (2001) found no significant differences in aflatoxin levels between Bt and non-Bt maize, but the Bt hybrids suffered less severe FAW infestations. These data indicate that insect damage is strongly correlated with fumonisin levels but not aflatoxin levels, suggesting that other factors such as drought stress and individual hybrid vulnerability may play a more dominant role than insect damage in the determination of aflatoxin levels. The field experiments concerning aflatoxin levels in Bt and non-Bt hybrids are summarized in Supplementary Table S2.

2.5. Contamination with other mycotoxins in Bt and non-Bt maize

The results obtained with other mycotoxins are also controversial. Significantly lower levels of DON were observed in Bt compared to non-Bt hybrids in some studies (Magg *et al.*, 2002; Schaafsma *et al.*, 2002; Selwet, 2011; Valenta *et al.*, 2001), whereas in other cases the mycotoxin levels appeared to be location-dependent (Bakan *et al.*, 2002; Papst *et al.*, 2005; Pinson *et al.*, 2002) or there was no difference between Bt and non-Bt hybrids (Barros *et al.*, 2009). A few studies have even found evidence for slightly higher DON levels in Bt hybrids, although the location was an important confounding effect (Folcher *et al.*, 2010; Bakan *et al.* 2002). Schaffsma *et al.* (2002) analyzed 102 commercial maize fields in Canada, reporting a reduction in DON levels in Bt hybrids depending on the severity of ECB infestation in each field. This was supported by a study carried out in Germany showing a reduction in DON levels in Bt compared to non-Bt hybrids (Valenta *et al.*, 2001). In another study, also in Germany, Magg *et al.* (2002) found significantly lower concentrations of DON in Bt maize in one of the two years of the experiment. Pinson *et al.* (2002) described differences in DON and ZEA levels between plots in two different fields in south and central France. Lower levels were observed in two Bt hybrids, but two others contained significantly higher levels of both DON and ZEA compared to the corresponding non-Bt cultivars. Bakan *et al.* (2002) reported low ZEA levels in their study, but significantly higher concentrations were observed in a traditional cultivar in France. In all the studies, the Bt hybrids expressed *cry1Ab* (Supplementary Table S3).

Only one study has investigated the impact of Bt on MON levels, and the Bt hybrids showed significantly lower levels of MON than non-Bt hybrids (isogenic and commercial hybrids) when infested with ECB. MON levels were significantly higher following the manual infestation of unprotected plants (296 μg/kg) compared to those treated with insecticide (66.2 μg/kg). In the infested plots, the MON concentrations were 153.5, 336.7 and 266.1 μg/kg for the transgenic, isogenic and commercial hybrids, respectively. In contrast, in the protected plots, the MON concentrations were 49.1, 99.3 and 42.9 μg/kg for the transgenic, isogenic and commercial hybrids, respectively (Magg *et al.*, 2003).

2.6. Economic impact of mycotoxin reduction in Bt maize

The main goal of Bt technology is to reduce pest damage and promote higher yields. However, indirect benefits such as the reduction of fumonisin levels also increase the percentage of maize grain that meets US and/or EU regulatory limits, which can have a significant economic impact and may also reduce the prevalence and severity of human and animal diseases (Bowers *et al.*, 2013; Folcher *et al.*, 2010; Hammond *et al.*, 2004; Magg *et al.*, 2002, 2003; Munkvold *et al.*, 1997, 1999; Pinson *et al.*, 2002).

Estimations for the cost of crop losses due to mycotoxin contamination in the USA range from $US 500,000 to $US 1.5 billion, reflecting variations in contamination levels, regulatory limits, price variations and production outputs (Vardon *et al.*, 2003). However, in the USA most losses are regulatory in nature (i.e. based on the rejection of grain based on quality) rather than actual harvest losses, and maize grains rejected for food and feed use may still be suitable for industrial processes such as biofuel production. On the other hand, the economic benefit of Bt maize in the USA has been specifically valued at $US 8.8 million in terms of preventing losses caused by fumonisins, and similarly $US 8.1 million for DON and $US 14 million for aflatoxins, even though the AF levels depend on the predominant pest species (Wu, 2006, 2007; 2014; Wu *et al.*, 2004). These data based on studies carried out over a decade ago do not take into account the increased adoption of Bt maize, which has risen from 30% in 2005 to more than 80% in 2015 (USDA-ERS, 2015). A recent study of the Thailand maize market estimated the economic losses due to aflatoxins. A loss of US$ ~6.9 million per annum was estimated assuming low levels of aflatoxin contamination (data from harvest and dried maize supplied by a pet company) but this increased to US$ ~100 million per annum assuming higher aflatoxin levels (data from retail markets). The rejection of aflatoxin-contaminated maize by the livestock sector is the most influential factor contributing to economic losses. Thus, the selection of high-quality maize (by the pet company) reflects lower levels of aflatoxin contamination, and lower economic losses (Lubulwa *et al.*, 2015). Bt technology can improve the quality of maize by reducing mycotoxin levels as an indirect consequence of preventing infestations with insect pests. This technology also reduces the need for chemical insecticides, resulting in lower levels of pesticide residues in food and water and less environmental impact (Qaim, 2009; Brookes and Barfoot, 2013).

2.7. Alternative strategies to reduce mycotoxin levels in maize

Although targeting pest insects can help to reduce opportunistic fungal infections of maize, other transgenic approaches have emerged more recently in which the fungus itself is the target. For example, Kant *et al.* (2012) reported a field study of transgenic maize expressing a modified rice *Rp13* gene encoding ribosomal protein L3, a primary target of DON. They developed two transgenic maize lines expressing the *Rp13* gene, one using the constitutive CaMV 35S promoter and the other using the silk-specific ZmGRP5 promoter. Both plants were less susceptible to *F. graminearum* than wild-type plants, and those containing the silk-specific promoter were the most tolerant, with the mildest symptoms under field conditions (DON levels were not evaluated). The difference in efficacy may reflect the broader activity of the silk promoter in the seed pericarp tissue. Maize silks are the primary route used by *F. graminearum* to infect the kernels. Expression of the modified *Rpl3* gene in silk tissue may therefore help to reduce *Gibberella* ear rot and hence DON levels.

Maize plants expressing the α-amylase inhibitor protein from *Lablab purpurea* (AILP) can also be used to reduce fungal infection. Fungal amylases liberate fermentable sugars from kernel starch which are essential for mycotoxin production. Kernel screening assays in AILP-transgenic maize plants revealed aflatoxin levels 56% lower than controls. AILP expression therefore appears to reduce both fungal growth on the kernels and aflatoxin accumulation (Chen *et al.*, 2015).

Another promising approach to reduce mycotoxin contamination is enzymatic mycotoxin detoxification *in situ*, which converts the mycotoxins into less toxic compounds. The ZEA lactone ring is sensitive to hydrolysis by the fungus *Clonostachys rosea*, which synthesizes an alkaline lactonohydrolase responsible for detoxification (Kimura *et al.*, 2006; Takahashi-Ando *et al.*, 2002). The corresponding gene (*zhd101*) was able to reduce ZEA levels *in vitro* and in field-grown plants compared to non-transgenic controls, even when infected with *F. graminearum*. The ability of transgenic seeds to degrade ZEA was evaluated by immersing the seeds in 50 μg/ml ZEA for 48 h. The kernel tissues were then analyzed by HPLC, showing that wild-type seeds contained 24.6 ± 1.7 μg ZEA/g whereas the transgenic seeds contained only 1.6 ± 0.4 μg ZEA/g. The detoxification of ZEA in *Fusarium*-infected transgenic kernels was evaluated after inoculation with *F. graminearum*. The wild-type seeds contained 15.4 ± 3.7 ng ZEA/g

whereas the level of ZEA in non-inoculated seeds and inoculated transgenic seeds was below the detection threshold (Igawa *et al.*, 2007). Similarly, the yeasts *Exophiala spinifera* and *Rhinocladiella atrovirens*, and the Gram-negative bacterium ATCC 55552, can produce enzymes that metabolize fumonisins. Duvick *et al.* (2003) patented a fumonisin esterase produced by these yeasts which can hydrolyze the tricarballylate esters of FB_1, and this is active in transgenic maize. The esterase gene reduced fumonisin levels in *Fusarium*-infected grain from 1.522 mg/kg without the enzyme to 0.379 mg/kg in esterase positive plants. Aflatoxin detoxification in transgenic plants has yet to be reported (Duvick, 2001; Hartinger and Moll, 2011; Jard *et al.*, 2011).

3. Concluding remarks

Several transgenic strategies can be used to reduce mycotoxin contamination in food and feed but Bt maize hybrids are widely grown and several studies have confirmed the reduction in pest damage, disease symptoms and fumonisin levels, particularly when ECB is the predominant pest. This is because fumonisin levels are reduced in Bt hybrids if the *Fusarium* population is dominated by species whose colonization of the plant is promoted by ECB damage (Miller, 2001).

Among the commercial Bt hybrids, Mon810 and Bt11 (which express *cry1Ab*) were associated with a reduction in the occurrence of *Fusarium* ear rot and fumonisins due to the lower level of kernel damage caused by susceptible lepidopteran pests (Dowd, 2000, 2001; Magg *et al.*, 2001; Munkvold and Hellmich, 1999; Papst *et al.*, 2005). Conversely, no difference has been reported between Bt 176 hybrids (Bt event 176 was withdrawn in 2001) and non-Bt maize (Magg *et al.*, 2002; Schaafsma *et al.*, 2002), and it has even been proposed that resistance against ECB and *Fusarium* ear rot may be inherited independently (Magg *et al.*, 2002; Miller, 2001).

The pest species and its abundance are key determinants of mycotoxin levels, particularly if the main pest is CEW which is unaffected by Cry1Ab (Clements *et al.*, 2003; Dowd, 2000; Hammond *et al.*, 2004). Lower levels of aflatoxins occur in Bt maize if the main pest is SWCB or CEW, but there is no difference between Bt and non-Bt maize if the pest is FAW (Buntin *et al.*, 2001; Williams *et al.*, 2002, 2005, 2006, 2010). When other pests are present, hybrids expressing Cry1Ab are inefficient and must be combined with further Cry proteins (such as Cry1F) or Vip proteins to increase the level of protection (Bowers *et al.*, 2013; 2014). Thus, if Bt events are not selected by taking into account prevalent insect pests and environmental conditions in

each field, Bt technology will not be effective. Hence the importance of Bt hybrids expressing multiple genes, whose performance in the presence of different pests and climate conditions has yet to be studied in detail.

The impact of Bt maize on the accumulation of aflatoxins, DON and ZEA is inconclusive because the extent of contamination depends on many interrelated factors. The different fungal infection pathways must be considered to understand the effect of Bt hybrids. The most common route used by *F. verticillioides* to infect the kernel is through the silks or through wounds caused by insect pests, whereas *F. graminearum* primarily reaches the kernels via the silks (Munkvold, 2003; Munkvold and Desjardins, 1997). *A. flavus* can infect maize kernels through the silks too. This may explain why Bt maize hybrids that are resistant to insect pests have less *Fusarium* ear rot and lower fumonisin levels, whereas the relationship with DON, ZEA and aflatoxin levels is not so clear cut. Further studies are necessary to determine the interaction between Bt maize and the different fungal species that produce these toxins.

Mycotoxin contamination is frequently linked with drought, heat stress and insects. Drought favors the accumulation of fumonisins more than heat stress (Miller, 2001). A 2-year study was carried out by Traore *et al.* (2000) to characterize the effect of drought stress on Bt maize. Water deficit during the vegetative period delayed leaf emergence, reduced the leaf area and caused stunted growth in both Bt and non-Bt hybrids. It also reduced grain and biomass yields and kernel number per ear during both years. However, the Bt hybrids had greater biomass in 1997 and greater grain yields in 1998 because they were not so severely infected by second-generation ECB. Therefore, Bt maize continues to play an important role in insect resistance under drought stress.

The first commercially available drought-tolerant GM maize variety (MON87460) expresses a bacterial cold shock protein B (CspB), a molecular chaperone derived from *Bacillus subtilis,* which may provide a yield advantage under limited water availability. A recent field study confirmed the higher grain yield of MON87460 under drought conditions compared to a conventional hybrid (Nemali *et al.*, 2015). The combination of Bt and drought-tolerant maize should therefore achieve even higher yields and lower mycotoxin levels because it will be protected against two major environmental stress factors.

Studies that considered aflatoxins and fumonisins simultaneously reported variable results, suggesting that diverse environmental conditions may

prevent the control of both mycotoxins in the same crops (Abbas *et al.*, 2002, 2006, 2007, 2008; Bruns and Abbas, 2006). More studies are needed to determine whether aflatoxin resistance traits can be crossed into Bt hybrids. Aflatoxin-resistant germplasm tends to possess undesirable agronomic traits such as tight husk coverage and late maturity. Breeding programs aiming to achieve the introgression of aflatoxin resistance into Bt hybrids could remove these undesirable characteristics while reducing aflatoxin contamination (Williams *et al.*, 2008; Williams *et al.*, 2010). Bt maize has a greater economic impact on *Fusarium* mycotoxins than aflatoxins (Wu, 2006, 2007; Wu *et al.*, 2004). Further studies are needed to evaluate the effect of both Bt hybrids expressing multiple genes and Bt hybrids combined with maize lines that are resistant to the accumulation of other mycotoxins, especially aflatoxins.

A potential risk that must be borne in mind comes in the form of mycotoxin derivatives (modified mycotoxins) that escape routine analytical techniques but may be digested by animals triggering toxic effects comparable to free mycotoxins. These derivatives should be included in the total mycotoxin allowances, and future legislation must consider their presence even though this would increase the stringency of testing and rejection, resulting in further economic loss. This highlights the benefits of Bt maize, which would reduce the levels of mycotoxins and potentially their derivatives, although the impact of Bt hybrids on the accumulation of modified mycotoxins needs to be addressed in more detail (De Boevre *et al.*, 2012, 2014; De Saeger and van Egmond, 2012; Wu, 2006). Finally, the development of transgenic plants expressing genes that protect against fungal infection (e.g. the modified *Rp13* gene and the AILP transgene) or reduce mycotoxin levels by *in situ* detoxification (e.g. *zhd101* and fumonisin esterase) could provide an additional strategy to control mycotoxins (Chen *et al.*, 2015; Duvick, 2001; Duvick *et al.*, 2003; Igawa *et al.*, 2007; Kant *et al.*, 2012) and could be combined with Bt hybrids to provide additive or even synergistic protection against mycotoxin-producing fungal pathogens.

Supplementary tables

Table S1. Summary of studies comparing fumonisin levels in Bt and non-Bt hybrids.

Table S2. Summary of studies comparting aflatoxin levels in Bt and non-Bt hybrids.

Table S3. Summary of studies comparing deoxynivalenol levels in Bt and non-Bt hybrids.

Table S1. Summary of studies comparing fumonisin levels in Bt and non-Bt hybrids. FB, fumonisin.

Bt events tested	Cry proteins expressed	Year (no. locations)	Location	Mold inoculation	Insect infestation	Area	FB levels (mg/kg) in Bt hybrids[b]		FB levels (mg/kg) in non-Bt hybrids[b]		Reference
Mon810 Bt11 Bt176 DBT418 CBH351	Cry1Ab Cry1Ab Cry1Ab Cry1Ac Cry9C	1995 (1)	USA (Iowa)	Natural	Natural	-	FB_1	≈ 2-2.5	FB_1	≈ 1	Munkvold *et al.* (1999)[d]
					Manual: ECB[a]	-	FB_1	≈ 3-6.5	FB_1	≈ 9	
		1996 (1)	USA (Iowa)	Natural	Natural	-	≈ 0.3-2.7		≈ 0.7-2.7		
					Manual: ECB[a]	-	≈ 1.7-5.5		≈ 3-12		
		1997 (1)	USA (Iowa)	Natural Manual: *F. verticillioides*	Natural	-	≈ 2-13		≈ 4-19		
					Manual: ECB[a]	-	≈ 2-12		≈ 10.5-24		
-	Cry1Ab	1997(3)	Northern Italy	Natural	Natural	-	1.97		20.05		Masoero *et al.* (1999)
Mon810	Cry1Ab	1997 (3)	Northern Italy	Natural	Natural	-	2.02		19.76		Pietri and Piva (2000)
		1998 (4)	Northern Italy	Natural	Natural	-	5.45		31.63		
		1999 (30)	Northern Italy	Natural	Natural	-	1.39		3.90		
Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	1996 (1)	USA (Illinois)	Natural Manual: *A. flavus*, *F. graminearum*	Natural	Peoria	< 0.35		< 0.35		Dowd (2000)
					Manual: ECB[a]	Peoria	2		8.8 ± 6		
		1997 (1)	USA (Illinois)	Natural Manual	Natural Manual: ECB[a]	Peoria	2.8 ± 1.5		4.8 ± 1.9		
		1998 (1)	USA (Illinois)	Natural Manual	Natural Manual: ECB[a]	Peoria	Not evaluated		Not evaluated		

Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	1998 (1)	USA (Illinois)	Natural	Natural	Manito	II	0.022	I	0.4	Dowd (2001)[c]
						Easton	II IV V	0.74 0.37 0.6	III	1.08	
		1999 (1)	USA (Illinois)	Natural	Natural	Kilbourne	VII	0.16	VI	0.5	
						Bath	II IV	1.08 0.42	-	-	
						Easton	II VIII	0.89 0.14	-	-	
Mon810	Cry1Ab	2000-2001 (2)	USA (Illinois)	Natural Manual: *F. verticillioides, F. graminearum*	Natural Manual: ECB[a], CEW[a]	Urbana and Monmouth	8 ± 13		10 ± 20		Clements *et al.* (2003)
Mon810	Cry1Ab	2000-2002 (several locations)	USA	Natural	Natural Manual: ECB[a]	Field trials	≈0.2-2		≈2-15.8		Hammond *et al.* (2004)[d]
Mon810 Bt176	Cry1Ab Cry1Ab	2001 (3)	East and South Germany	Natural	Natural	-	Not detected		I II	0.016 0.032	Papst *et al.* (2005)
					Manual: ECB[a]	-	0.569		I II	4.85 2.71	
Mon810	Cry1Ab	2000 (4)	Argentina	Natural	Natural	-	2.46		6.29		De la Campa *et al.* (2005)
		2001 (4)	Argentina	Natural	Natural	-	0.56		3.06		
			Phillippines	Natural	Natural	-	0.81		0.97		
		2002 (2)	Phillippines	Natural	Natural	-	0.25		0.45		
-	Cry1Ab	2002 (7)	Argentina	Natural	Natural	-	0.043		0.173		Barros *et al.* (2009)
		2003 (5)	Argentina	Natural	Natural	-	0.2		0.633		
Mon810	Cry1Ab	2005 (21)	Southwestern France	Natural	Natural	-	0.26		6.11		Folcher *et al.* (2010)
		2006 (21)	Southwestern France	Natural	Natural	-	0.43		5.62		

-	Cry1Ab Cry1Ab xVip3Aa	2008 (1) 2009 (1) 2011 (1)	USA (Iowa)	Natural	Natural	-	Cry1Ab Cry1Ab x Vip3Aa	2.2 0.61	4.23	Bowers *et al.* (2013)
					Manual: ECB[a]	-	Cry1Ab Cry1Ab x Vip3Aa	1.52 0.57	6.75	
					Manual: CEW[a]	-	Cry1Ab Cry1Ab x Vip3Aa	2.55 0.61	7.51	
					Manual: WBC[a]	-	Cry1Ab Cry1Ab x Vip3Aa	2.08 0.39	2.37	
Mon810 TC1507	Cry1Ab Cry1F	2008 (1)	USA (Iowa)	Natural	Natural	-	Cry1Ab Cry1F	1.45 1.26	0.87	Bowers *et al.* (2014)
					Manual: ECB[a]	-	Cry1Ab Cry1F	1.4 1.99	12.34	
		2009 (1)			Natural	-	Cry1Ab Cry1F	0.15 0.04	0.12	
					Manual: ECB[a]	-	Cry1Ab Cry1F	0.3 0.2	1.25	
					Manual: WBC[a]	-	Cry1Ab Cry1F	0.78 0.23	0.64	
		2010 (1)			Natural	-	Cry1Ab Cry1F	0.29 0.61	0.42	
					Manual: ECB[a]	-	Cry1Ab Cry1F	0.69 0.56	1	
					Manual: WBC[a]	-	Cry1Ab Cry1F	0.55 0.12	0.86	

[a] ECB: European corn borer; CEW: Corn earworm; WBC: Western bean cutworm.

[b] Roman numerals indicate maize varieties, but they are different among the studies.

[c] Study in commercial fields, the remainder are field experiments.

[d] The data that correspond with this study have been interpreted from a graphic.

Table S2. Summary of studies comparing aflatoxin levels in Bt and non-Bt hybrids. AF, aflatoxin.

Bt events tested	Cry proteins expressed	Year (no. locations)	Location	Mold inoculation	Insect infestation	Area	Treatment		AF levels (µg/kg) in Bt hybrids[b]	AF levels (µg/kg) in non-Bt hybrids[b]	Reference
Bt11	Cry1Ab	1995-1997 (1)	USA (Mississippi) (only Bt hybrid in 1997)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Day 7	*A. flavus* SWCB *A. flavus* + SWCB	166 4 83	45 19 136	Windham *et al.* (1999)
						-	Day 21	*A. flavus* SWCB *A. flavus* + SWCB	145 5 290	45 41 650	
						-	Control		17	6	
Mon810	Cry1Ab	1999 (2)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	CC	Dryland		1,136	601	Odvody *et al.* (2000)
						CC	Irrigated		423	243	
		2000 (3)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	CC	Dryland		1,399	1,166	
						CC	Irrigated		1,078	979	
						BEE	Dryland		187	347	
-	Cry1Ab	2000 (2)	USA (Indiana and Ilinois)	Natural Manual: *A. flavus*	Natural	-	-		Data not available	Data not available	Maupin *et al.* (2001)
Mon810	Cry1Ab	1998 (3)	USA	Natural	Natural	A	-		≈300	≈425	Buntin *et*

Bt11	Cry1Ab		(Georgia)			P	1st planting	≈875	≈700	*al.* (2001)[c]
							2nd planting	≈75	≈100	
							3rd planting	≈25	≈25	
Mon810 Bt11	Cry1Ab Cry1Ab	2000	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*	942	936	Williams *et al.* (2002)
							Spray *A. flavus*	311	646	
							Spray + SWCB	360	945	
							Control	115	328	
		2001 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*	530	808	
							Spray *A. flavus*	61	237	
							Spray + SWCB	138	408	
							Control	9	23	
Mon810 Mon851 Mon840 Mon84006	Cry1Ab Cry1Ab Cry2Ab Cry2Ab	2000-2001 (2)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	-	-	Data not available	Data not available	Odvody *et al.* (2002)
Mon810 Bt11 Bt176	Cry1Ab Cry1Ab Cry1Ab	2001-2002 (3)	USA (South Texas)	Natural Manual: *A. flavus*	Natural	-	-	Data not available	Data not available	Odvody *et al.* (2003)
-	Cry1Ab	2001 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle *A. flavus*	637	784	Williams *et al.* (2005)
							Spray *A. flavus*	140	192	
							Spray + SWCB	110	417	
							Control	15	24	
		2002 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	Needle	261	391	
							Spray	5	5	
							Spray + SWCB	5	22	
							Control	2	4	

-	Cry1Ab	1998 (1)	USA (North Florida)	Natural	Natural	-	-	314	Normal Tropical	634 470	Wiatrak *et al.* (2005)
		1999 (1)	USA (North Florida)	Natural	Natural	-	-	70	Normal Tropical	86 259	
		2000 (1)	USA (North Florida)	Natural	Natural	-	-	55	Normal Tropical	36 48	
-	-	1998 (1)	USA (Arkansas)	Natural	Natural	CB	-	196	227		Abbas *et al.* (2006)
		1999 (1)	USA (Arkansas)	Natural	Natural	CB	-	3.6	26.6		
		2001 (2)	USA (Arkansas)	Natural	Natural	CB	-	< 5	10		
						PT		< 5	6.5		
-	-	2002-2004 (1)	USA (Mississippi)	Natural	Natural	-	-	12.4 (data 2003)	45.3 (data 2003)		Bruns and Abbas (2006)
-	Cry1Ab	2003-2005 (1)	USA (Mississippi)	Natural Manual: *A. flavus*	Natural Manual: SWCB[a]	-	-	I II	10 4	I II	57 33	Williams *et al.* (2006)
-	-	2002 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April Planting in May	0.18 11.5	1.3 0.28			Abbas *et al.* (2007)
		2004 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April Planting in May	1.1 75.7	17 20			
		2005 (1)	USA (Arkansas)	Natural	Natural	CB	Planting in April	2.1 12.9	5.2 8.5			

							Planting in May			
Mon810	Cry1Ab	2006 (1)	USA (Mississippi)	Natural	Natural	-	-	109	211	Abbas *et al.* (2008)
-	Cry1Ab	2009 (3)	USA (North Carolina)	Manual: *A. flavus*	Natural	-	-	249	382	Williams *et al.* (2010)
			USA (Georgia)	Manual: *A. flavus*	Natural	-	-	287	398	
			USA (Mississippi)	Manual: *A. flavus*	Natural	-	-	259	332	
-	Cry1Ab	2008 (1)	USA (Mississippi)	Natural	Natural	-	No-till	775	2,381	Abbas *et al.* (2013)
							Tillage	272	266	
		2009 (1)	USA (Mississippi)	Natural	Natural	-	No-till	631	1,457	
							Tillage	755	2,381	
Mon88017+ Mon89034 GA21 TC1507	Cry1A.105 Cry2Ab CP4 EPSPS Cry3Bb CP4 EPSPS CP4 EPSPS PAT Cry1F	2010-2012 (1)	USA (Mississippi)	Manual: *A. flavus*	Natural	-	-	Data not availa-ble	Data not available	Bruns and Abbas (Un-published data)

[a] SWCB: Southwestern corn borer.; [b] Roman numerals indicate maize varieties, but they are different among the studies; [c] The data that correspond with this study have been interpreted from a graphic.

Table S3. Summary of studies comparing deoxynivalenol (DON) levels in Bt and non-Bt hybrids.

Bt events tested	**Cry proteins expressed**	**Year (no. of locations)**	**Location**	**Mold inoculation**	**Insect infestation**	**Area**	**DON levels (µg/kg) in Bt hybrids[b]**		**DON levels (µg/kg) in non-Bt hybrids[b]**		**Reference**
Mon810	Cry1Ab	1999 (12)	South Germany	Natural	Manual: ECB[a]	-	152		873		Valenta *et al.* (2001)
Bt176	Cry1Ab			Natural	Natural		51		77		
Mon810	Cry1Ab	1999 (5)	South-western France	Natural	Natural	O25	729		472		Bakan *et al.* (2002)
						O30	332		751		
						O32	181		179		
			Northern Spain	Natural	Natural	SP1	17		82		
						SP2	20		271		
Mon810	Cry1Ab	1999 (4)	Germany (Upper Rhine Valley)	Natural	Natural Manual: ECB[a]	-	69		I II	138 136	Magg *et al.* (2002)
Bt176	Cry1Ab	2005 (5)	Germany (Upper Rhine Valley and Bavaria)	Natural	Natural Manual: ECB[a]	-	717		I II	867 765	
Bt11	Cry1Ab	1996-1999 (102)	Canada (Ontario)	Natural	Natural	-	0.57 µg/g		0.96 µg/g		Schaafsma *et al.* (2002)[c]
Mon810	Cry1Ab	France 2000 (4)	Southern France	Natural	Natural	32	I II	2 4	I II	5 3	Pinson *et al.* (2002)
				Natural	Natural	82	I II	10 123	I II	38 43	
			Central France	Natural	Natural	41	III IV	5 5	III IV	10 12	
				Natural	Natural	45	III IV	37 14	III IV	110 23	

Mon810	Cry1Ab	2008 (1)	Poland (Wroclaw)	Natural	Natural	-	I	41	II III IV V	213 254 198 201	Selwet (2011)
		2009 (1)	Poland (Wroclaw)	Natural	Natural	-	I	32	II III IV V	201 243 186 199	

[a] ECB: European corn borer.

[b] Roman numerals indicate maize varieties, but they are different among the studies. The results are expressed in μg/kg, except in one study, Schaafsma *et al.* (2002), where are expressed in μg/g.

[c] Study in commercial fields, the remainder are field experiments.

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