Conjugated linolenic acids of plant origin: bioconversion in laying hens, enrichment of egg yolk and impact of their consumption on human health

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À Maman

À l'Ancien des jours, qui a commencé cette œuvre et a été fidèle pour la mener à son accomplissement.

Les cieux racontent la gloire de Dieu, et l'œuvre de ses mains, le firmament l'annonce;
le jour au jour en publie le récit et la nuit à la nuit transmet la connaissance.

Non point récit, non point langage, nulle voix qu'on puisse entendre,
mais pour toute la terre en ressortent les lignes et les mots jusqu'aux limites du monde.

Là-haut, pour le soleil il dressa une tente,
et lui, comme un époux qui sort de son pavillon, se réjouit, vaillant, de courir sa carrière.

À la limite des cieux il a son lever et sa course atteint à l'autre limite, à sa chaleur rien n'est caché.

La loi de l'Éternel est parfaite, réconfort pour l'âme;
le témoignage de l'Éternel est véridique, sagesse du simple.

Les préceptes de l'Éternel sont droits, joie pour le cœur;
le commandement de l'Éternel est limpide, lumière des yeux.

La crainte de l'Éternel est pure, immuable à jamais;
les jugements de l'Éternel sont vérité, équitables toujours,

désirables plus que l'or, que l'or le plus fin; ses paroles sont douces plus que le miel, que le suc des rayons.

Aussi ta servante s'en pénètre, les observer est grand profit.

Mais qui s'avise de ses faux pas? Purifie-moi du mal caché.

Préserve aussi ta servante de l'orgueil, qu'il n'ait sur moi nul empire! Alors je serai irréprochable et pure du grand péché.

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Summary

In addition to providing nutrients, certain food items contain specific concentrations or ratios of biologically active compounds that have a significant influence on physiological functions and processes. Several health benefits have been observed with omega-3 fatty acids, including alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA), which play an important role in the development and maintenance of different organs, mainly the brain, and could be useful in the prevention of cardiovascular diseases and certain cancers. Beside them, rumenic acid (RmA), an omega-7 conjugated linoleic acid (CLA) and its conjugated linolenic acid (CLnA) precursors, α-eleostearic acid (α-ESA) and punicic acid (PunA), are suspected of having a large set of biological activities such as antidiabetic, anti-inflammatory and anticancer properties. Humans cannot or insufficiently synthesize these fatty acids that must be supplied through the diet. In this regard, the development of a widely consumed food enriched in ALA, DHA, RmA and related CLnA may be a solution to provide the human diet with these health-promoting fatty acids.

Hen's egg holds a special place as a cheap and well-balanced source of high-quality amino acids and essential fatty acids, as well as several minerals and vitamins. In addition, its multifunctional attributes make it an ingredient in many food formulations. In the present work, we improved the fatty acid profile of eggs through an improvement of hens' diet. We fed laying hens with a flaxseed-rich feed, combined with a CLnA-rich seed oil, either from *Ricinodendron heudelotii* or *Punica granatum*. Eggs produced contained up to 165 mg of ALA, 100 mg of DHA and 650 mg of RmA. They also contained CLnA, at levels depending on the CLnA-rich oil used. Consumption by men and women of two eggs enriched in omega-3 fatty acids, CLA and CLnA per day for three months resulted in reducing their abdominal obesity. These eggs are thus proposed as functional foods to be incorporated in a healthy and balanced diet for disease prevention.

Contents

Abbreviations

AI	Augmentation index
ALA	α-Linolenic acid
ALT	Alanine aminotransferase
ARA	Arachidonic acid
AST	Aspartate aminotransferase
BMI	Body mass index
CLA	Conjugated linoleic acid
CLnA	Conjugated linolenic acid
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
eGFR	Estimated glomerular filtration rate
EPA	Eicosapentaenoic acid
ESA	Eleostearic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FS	Flaxseed
FSH	Follicle stimulating hormone
GGT	Gamma-glutamyltranspeptidase
GnRH	Gonadotropin-releasing hormone
HbA1c	Glycosylated hemoglobin
HbNO	Nitrosylated hemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment for insulin resistance
hs-CRP	High-sensitivity C-reactive protein
IL-6	Interleukin 6
LA	Linoleic acid

LDL	Low-density lipoprotein
LH	Luteinizing hormone
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acid
NO	Nitric oxide
OA	Oleic acid
OL	Olive oil
Ox-LDL	Oxidized low-density lipoprotein
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor
PSO	Pomegranate seed oil
PUFA	Polyunsaturated fatty acid
PunA	Punicic acid
QUICKI	Quantitative insulin sensitivity check index
RHI	Reactive hyperaemia index
RHK	*R. heudelotii* kernel
RHO	*R. heudelotii* oil
RmA	Rumenic acid
ROS	Reactive oxygen species
SFA	Saturated fatty acid
TG	Triglyceride
TNFα	Tumor necrosis factor alpha
VLDL	Very low-density lipoprotein
VLDLy	Yolk-targeted very low-density lipoprotein
WHR	Waist-to-hip ratio

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Introduction

The hen's egg is a food of great nutritional value in human diet. It is an important source of lipids and proteins, and also contains many vitamins and minerals. However, some elements of its composition are responsible for a negative image sometimes perceived by consumers. In recent years, the egg has received unfavorable publicity, which was largely attributed to its high cholesterol content. Extensive research of the last decade has now shown that dietary cholesterol does not necessarily influence serum cholesterol. Apart from that, researchers investigated strategies to improve the positive attributes of the egg and restore its position as a highly nutritious food that can offer specific benefits. According to data from FAO, the average egg consumption per capita reached 9.68 kg in 2018 worldwide[1]. This makes eggs one of the most widely consumed foods over the world.

Evidence that high dietary intake of omega-3 fatty acids helps to reduce the incidence of cardiovascular diseases and the recognition of these fatty acids as essential for humans have generated several research programs aiming to enrich the egg with omega-3 fatty acids. Flaxseed has been the preferred feed ingredient to increase omega-3 fatty acids in the egg, as it causes fewer off-flavor problems than marine-derived products. Omega-3 enriched eggs are now marketed worldwide and appear to be gaining increasing popularity among health-conscious consumers. Eggs are currently one of the preferred foods for enrichment. Several studies are being carried out to enrich eggs with different macro- or micronutrients. The success of these initiatives depends

[1] Source: Food and Agricultural Organization (FAO)
http://www.fao.org/faostat/en/#data/FBS

on several factors including the nature of the feed ingredient, the metabolization in the hen and the level of enrichment achieved.

This thesis reports on the improvement in the levels of conjugated linoleic acids (CLA) and conjugated linolenic acids (CLnA) in eggs by the feeding of laying hens with natural oils rich in CLnA, combined or not with flaxseeds. The first two Chapters (1 and 2) review the formation of the egg in the laying hen and its composition. Chapter 3 presents the health attributes of CLA and CLnA, as well as some results of work aiming at increasing their concentrations in food products. The specific objectives of the thesis are set in Chapter 4. The experimental part of the thesis includes Chapters 5, 6 and 7, which are articles published in international peer-reviewed journals. All of this work is discussed in Chapter 8, and Chapter 9 presents the general conclusions with some prospects for future investigations.

Chapter 1

Reproduction in hens and egg formation

1.1. Introduction

The class of birds includes higher tetrapod vertebrates whose upper limbs have evolved into wings to allow flight. Various anatomical and physiological features, such as aerodynamic body, strong pectoral musculature, feathers and airbags, accompany the flight capability. Birds are warm-blooded endothermic animals. Besides, the development of a beak wrapping a toothless jaw has led to the evolution of a specially adapted digestive system. Birds are oviparous animals with internal fertilization. This oviparity presents many specificities and distinguishes them from other oviparous vertebrates.

Unlike mammals, one of the characteristics of birds is that the female is the heterogametic sex. The sex chromosomes Z and W are found as ZZ homogametic pair in the male and ZW heterogametic pair in the female. Anatomical features such as the existence of a single functional ovary and oviduct characterize female reproduction. Female birds lay enormous telolecithal eggs containing a large amount of yolk with a thin and hard shell, which provide the embryo with a very secure nourishing environment. In addition, the brooding behaviour leads to the maintenance of a temperature favouring embryonic development. These particularities have greatly facilitated the improvement of poultry production and have contributed to making poultry an essential source of animal protein for human consumption.

The development of the poultry industry in the 20th century has led to the genetic selection of two types of animals. "Laying" or "light" chickens, which produce eggs for consumption, have been selected for reproductive efficiency. "Broiler" or "heavy" chickens with lower reproductive performance than laying chickens are bred for meat production, because their selection relates to carcass growth. This chapter deals with reproduction in chicken (the systematic classification is presented below), anatomical structures allowing the formation of the egg, as well as associated

physiological phenomena. Unless otherwise indicated, the reported information relates to observations made in the laying hen.

Gallus gallus domesticus (Linnaeus, 1758)	
Kingdom:	Animalia
Phylum:	Chordata
Class:	Aves
Order:	Galliformes
Family:	Phasianidae
Genus:	*Gallus*
Species:	*gallus*
Subspecies:	*domesticus*

1.2. The female reproductive system

In hens, as in most of the females of the Galliformes avian order, the reproductive system consists of two essential parts: a single left ovary and its oviduct. The right ovary and oviduct present in young hens regress before hatching (Leborgne et al., 2014). The ovary and the different parts of the oviduct of an adult hen are represented in Figure 1.1.

1.2.1. The ovary

The ovary is located in the upper part of the abdomen. It relies on the kidney and lung, and ventrally, on the left abdominal air sac. The ovary consists of two regions, an outer envelope or cortex that surrounds a highly vascularized central part called medulla. In the medullary zone are the connective tissue, the blood and lymphatic vessels. The cortical area contains the ovarian

follicles. The ovary performs two functions: a reproductive function by the production of gametes and an endocrine function related to the production of hormones (Leborgne et al., 2014; Sauveur, 1988).

At hatching, the ovary of the juvenile breeder female is undeveloped and contains mainly connective tissue (stroma). It weighs only about 300 mg. As the female approaches sexual maturity, the cortex becomes more granular. This change in appearance is related to the maturation of the follicles. The smallest follicles contain «white» yolk. When they are recruited into the large follicle pool, they become yellow (Robinson & Renema, 2003). The ovary then evolves from light grey to bright yellow in the last weeks before sexual maturity. The mature ovary weighs between 60 and 70 g in a laying hen, but can reach 120 g or 150 g in a hen fed *ad libitum*. The follicles then have a size hierarchy to a greater or lesser degree depending on the age of the hen (Leborgne et al., 2014).

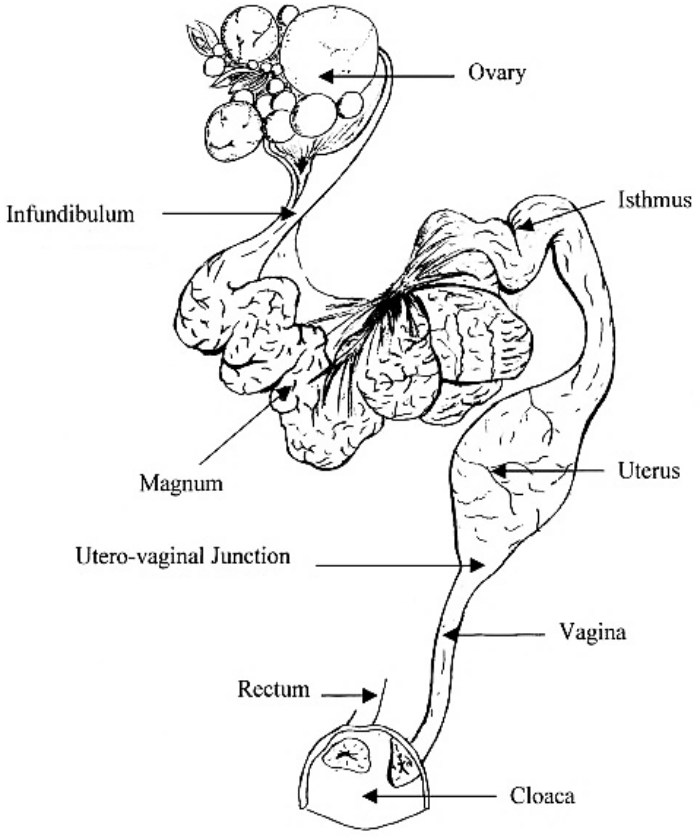


Figure 1.1. Representation of a functional oviduct and ovary in an adult hen (Bréque, Surai, & Brillard, 2003)

The follicular pool is established before the chicks hatch. Several thousand small follicles (1,000 – 12,000 or more) are present in the ovary, but only a part (250 – 500) will complete development and lead to the production of yolk. The ovary of an adult hen in excellent laying condition has the appearance of a cluster due to the presence of 5 – 6 follicles, whose yolk is in rapid growth phase and next to which there are many small follicles, as well as one or two empty post-ovulatory follicles (Figure 1.2).

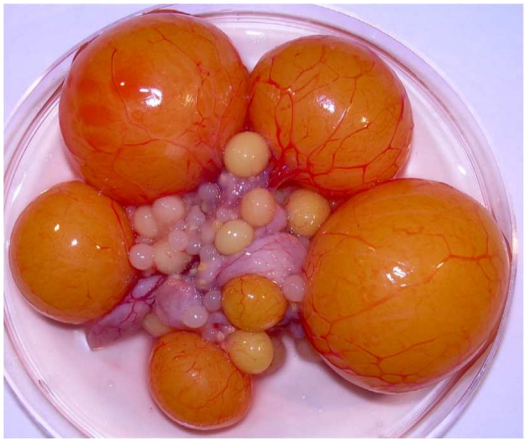


Figure 1.2. Adult hen ovary and its ovarian cluster (Johnson, 2015). The growing size of the follicles shows a follicular hierarchy. The largest follicle will ovulate in the next 24 hours, the next around 48 hours, etc.

Sexual maturity occurs in the hen around 5 months of age when reared under a non-stimulating photoperiod, i.e. less than 12 hours of light per day. But, photostimulation can significantly advance maturity to an earlier age (Johnson, 2015). The factors that determine when a hen will reach sexual maturity are not clearly defined. Hens are expected to meet a minimum threshold of body weight to start laying. Some strains reach sexual maturity earlier when they are fed *ad libitum* than when they are in restricted feeding. However, other strains do not start earlier when they are fed *ad libitum*, which suggests the need to reach a certain age. This requirement probably deals with the maturation of the hypothalamus to release Gonadotropin-releasing hormone (GnRH) appropriately (Robinson & Renema, 2003).

The hypothalamus releases GnRH, which in turn induces the anterior pituitary gland to produce gonadotropin hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). The anterior pituitary gland is strictly essential for the development and maintenance of ovarian activity. Its ablation leads to gonad atrophy. Under the control of pituitary gonadotropic

hormones FSH, LH and prolactin, the ovary secretes the three main sex steroids: oestrogens, androgens and progesterone. This secretion is partly cyclic in relation to ovulation but also ensured at a very low level continuously (Sauveur, 1988).

1.2.2. Anatomy of the oviduct

The oviduct is located in the left lateral position, folded up against the wall of the abdomen. It is suspended from the abdominal ceiling in the region of the left kidney by two thick ligaments enclosing smooth muscle fibres. The oviduct is the place of formation of the egg. After the hatching, the morphological development of the oviduct follows that of the ovary. As sexual maturity approaches, the oviduct grows rapidly from 15 to 70 cm long. It then appears as a long, pale pink tube. From the ovary to the cloaca, one distinguishes successively: the infundibulum, the magnum, the isthmus, the uterus, the utero-vaginal junction and the vagina (Figure 1.1). It is only in sexual maturity that communication is established between the oviduct and the cloaca (Leborgne et al., 2014; Sauveur, 1988).

– The infundibulum is located in the upper part of the oviduct. Funnel-shaped, it is called the pavilion and catches the oocyte at the time of ovulation. The infundibulum is the place of fertilization of the oocyte. Its wall is thin and its internal mucosa contains several categories of cells. Among these cells, some have a secretory function and ensure the deposition of proteins that form the perivitelline membrane of the egg. There are also cells responsible for the storage of spermatozoa.

– The magnum is the richest zone in cells and secretory glands. It constitutes the region in which the albumen or egg white is synthesized and deposited. It measures 30 to 35 cm in the adult hen. Its wall is very extensible and has on its inner side very large folds whose thickness can reach 5 mm.

– The isthmus is 15 cm long and is slightly narrower than the magnum. Its internal folds are also less stressed. It is during the 1 to 2 hours passage of the egg through the isthmus that the shell membranes are formed.

– The uterus or shell gland is distinguished from previous segments by its pocket shape and its highly developed muscular wall. The dark red uterine mucosa is constituted by numerous folds covered with an epithelium strewed with tubular glands responsible for the secretion of the constituents of the shell. The egg remains in the shell gland for 18 to 20 hours (Leborgne et al., 2014).

– The utero-vaginal junction has a length of 1 to 2 cm and a flared shape narrowing in its lower part. It is attached to the uterus by a thick fibrous structure and plays an essential role in the progression and the preservation of sperm cells.

– The vagina is separated from the uterus by the utero-vaginal junction. Its inner wall presents longitudinal folds but does not include secretory glands. It is an important layer of muscle tissues that allows, in coordination with the shell gland, the expulsion of the egg or oviposition. The vagina opens into the urodeum, the middle part of the cloaca. The cloaca is divided into three compartments; the anterior is the copradeum followed by the urodeum and the proctodeum. During oviposition, a thin membrane is formed across the rectum by an urodeal fold to prevent the excretion of faeces (Johnson, 2015).

1.3. Vitellogenesis: formation of the yolk of the egg

In the hen, the formation of the egg consists of two different stages. The first takes place in the ovary and corresponds to the development of the oocyte before ovulation. The second step takes place after ovulation in the different anatomical regions of the oviduct and involves the sequential deposition on the oocyte of the perivitelline membrane, albumen, shell membranes and shell.

1.3.1. Oogenesis

Oogenesis begins on the 7th day of embryonic life. Primordial germ cells transform into oogonia and undergo repeated mitosis to give diploid primary oocytes (2n chromosomes). At hatching, the nucleus of the primary oocyte (oocyte I) is in prophase I of meiosis, precisely at the pachytene stage[2]. Then, it evolves to the diplotene stage where it remains blocked for months to years. Oocytes I (around 12,000) constitute the final stock of gametes and will not be renewed during the life of the hen. Only a few (250 – 500) will mature and ovulate during the life of the hen. (Johnson, 2015; Leborgne et al., 2014). The reductional division of the oocyte I occurs 24 hours before ovulation by the expulsion of the first polar body[3], producing the haploid oocyte II (n chromosomes). The female of the birds being heterogamous, the sex of the future embryo is therefore determined from this stage. In the case of fertilization, the equational division with the expulsion of the second polar body

[2] The prophase I of the reduction meiosis is subdivided into five stages, characterized by the chromosomal aspects observed during this stage: the leptotene, zygotene, pachytene, diplotene and diacinesis stages.

[3] Haploid cell with the same chromosomal equipment as oocyte II but no cytoplasm.

occurs after ovulation in the infundibulum, as a result of the penetration of the spermatozoon nucleus into oocyte II.

1.3.2. Folliculogenesis

The follicle consists of an oocyte surrounded successively from the inside to the outside by a perivitelline layer, a single-cell layer called granulosa, a basal lamina and two theca (internal and external) (Figure 1.3). Folliculogenesis is a long and continuous biological process that begins in the embryonic gonad and ends in the adult hen with ovulation. It consists of the growth and maturation of the follicles and is accompanied by an accumulation of vitellus (vitellogenesis) in the ovarian follicle. Folliculogenesis can be divided into three main phases, summarized in Table 1.1.

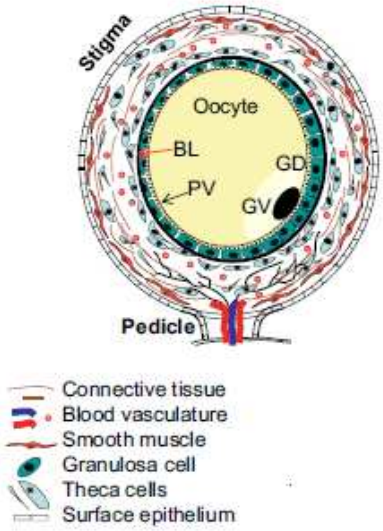


Figure 1.3. Structure of a mature follicle. The mature follicle consists of the germinal vesicle (GV) localized within the germinal disc (GD) contained within a perivitelline membrane (PV) and surrounded by granulosa cells. A basal lamina (BL) separates the granulosa cells layer from the internal and external theca layers (adapted from Johnson, 2015).

– **The initial phase of slow growth.** At the time of the hatching of the young female chick, the oocytes are in the cortical zone of the ovary and are 10 to 20 micrometres in size. Quite quickly, they surround themselves with layers of cells that represent the future follicular envelopes. At 6 weeks of age, the diameter of the oocytes is multiplied by four and reaches 1 mm around 4 to 5 months of age after deposition of lipid droplets. At this stage, follicular growth stops. Some follicles will be stuck there for months or years.

– **The intermediate growth phase.** After the selection of a follicle in the undifferentiated pool, its size increases from 1 to 4 mm in about 50 days, by a deposit consisting essentially of proteins and some lipids. The whole constitutes the white vitellus. During this phase, the oocyte migrates to the surface of the follicle, leaving a trace called latebra.

– **The rapid growth phase.** During the 7 to 11 days before ovulation, the growth of the follicle accelerates. It quickly accumulates a large amount of lipids and its weight increases from 200 mg to 15 – 18 g. The ovary of an adult hen contains 6 – 10 follicles in phase of rapid growth with an initial lag of one day. The order in which the follicles ovulate and the degree of their growth correspond to their recruitment. The follicular hierarchy is perfectly visible in the ovary. The largest follicle, called F1, ovulates first; it is followed by F2, F3, etc. This hierarchy may be disturbed with the aging of the animal because of the decrease in follicular activity. Ovarian follicles that initially begin to grow but do not reach the differentiated stage at which they are ovulated become atretic. The loss of viability and resorption of these follicles are programmed and occur by apoptosis. Likewise, apoptosis is rapidly induced in postovulatory follicles within 24 hours of ovulation (Tilly, Kowalski, Johnson, & Hsueh, 1991).

Table 1.1. Stages of folliculogenesis in hens (adapted from Sauveur, 1988)

Growth phase	Age	Duration	Follicle diameter	Stage of development	Deposited substances	Amount of follicles
Slow growth	1 day		10 – 20 µ	Cortical follicles	Lipid droplets	Thousands
	6 weeks		50 – 100 µ			
	18 weeks		1 mm			
Individual selection of follicles						
Intermediate growth		50 days	2 – 4 mm	Small white follicles	Proteins	Hundreds
Rapid growth		7 – 11 days	5 – 35 mm	Small yellow follicles growing in pre-ovulatory follicles	Lipids Lipoproteins Proteins Ca, Mg, Fe	6 – 10

1.3.3. Synthesis of the constituents of the yolk

With the approach of sexual maturity in the female and the onset of spawning, a series of metabolic changes take place. The rate of lipid synthesis from carbohydrates in the liver increases to such a high level that the total concentration of triacylglycerol in the liver increases by about 4-fold. Lipid synthesis results in an increase of more than 20-fold the plasma concentration of very low-density lipoprotein (VLDL). Similarly, plasma concentrations of vitellogenin, as well as certain other yolk-specific proteins such as certain vitamin-binding proteins, which are also synthetized by the liver, increase dramatically. The yolk-targeted VLDL (VLDLy) particles secreted by the liver of the laying hen are unusually small (about 30 nm in size) and contain two apoproteins, namely apoB (MW 500 kDa) and apoVLDL-II (MW 9.5 kDa) (Walzem, Hansen, Williams, & Hamilton, 1999). VLDLy already exist in the blood of immature pullet, but their production in the hen's liver increases considerably with sexual maturity. Increases in liver lipid and

lipoprotein production at the onset of egg-laying occur in a coordinated manner and are regulated by changes in plasma oestrogen concentration. Thus, the liver expression of genes coding for vitellogenin, apoVLDL-II, vitamin-binding proteins and lipogenic enzymes is induced or increased by oestrogen.

The VLDLy (86% lipid content by wt.) and vitellogenin (15 – 20% lipid content) are transported to the ovary via the bloodstream and incorporated into the developing oocyte. The lipid composition of VLDLy is not modified during uptake from the plasma into the oocyte. VLDLy have properties that promote their delivery to the ovary and their incorporation into the oocyte. Their small size is apparently a modification to facilitate uptake by the oocyte. The basal lamina acts as a filter preventing the passage of large lipoproteins such as intestinal portomicrons, the avian equivalent of chylomicrons, but allowing the transfer of smaller apoVLDL-II containing lipoproteins. Binding of VLDLy particles to specific receptors on the oocyte plasma membrane is followed by receptor-mediated endocytosis. The binding to the receptor occurs through the apoB component and not through apoVLDL-II. Vitellogenin is also absorbed into the oocyte via the same receptor (Speake, Murray, & Noble, 1998).

1.4. Egg formation in the oviduct

Unless otherwise indicated, the information presented in this section is taken from the book «Reproduction des animaux d'élevage» by Leborgne et al. (2014).

Ovulation is ensured by the opening of the follicle at the level of the stigma (Figure 1.3). Between the time of ovulation and that of laying, the oocyte is driven through the oviduct by peristaltic contractions, and matures through different stages, which correspond to the addition of constituents to form the complete egg (Table 1.2).

1.4.1. Deposition of the outer perivitelline membrane

The capture of the oocyte by the infundibulum is the first stage of the activity of the oviduct. The outer perivitelline membrane is formed from infundibular secretions, consisting of a layer of fibrils, having a composition very close to that of thick white. This deposit plays an important role in the protection of the yolk against water transfers from the albumen.

1.4.2. Secretion of the albumen

Albumen is essentially an aqueous solution of proteins and minerals. Its detailed composition is given in Chapter 2. The proteins of the albumen are continuously synthesized locally in the magnum. Their release occurs when the oocyte passes through the magnum. When the egg leaves the magnum, the albumen is in the form of a thick gelled mass, very viscous, low in water and minerals.

1.4.3. Formation of shell membranes

The forming egg reaches the isthmus about 3 hours 30 after ovulation. When it arrives, it begins to be covered with protein fibres, the interlacing of which will constitute the shell membranes. The secreted material comes mainly from tubular glands and the deposition takes place at a constant speed as the egg progresses through the isthmus. At the terminal portion of the isthmus called red isthmus, the constituent fibres of the lower part (mammillary layer) of the organic matrix of the shell are deposited. These fibres are nested in the outer shell membrane and ensure the attachment strength of the shell.

1.4.4. Formation and mineralization of the egg shell

The egg enters the uterus 5 to 6 hours after ovulation. It will remain there for another 20 hours before being expelled. At the exit of the isthmus, the egg covered with the two membranes has a wrinkled appearance due to the low hydration of the proteins of the white. The first uterine activity before mineralization of the egg is the hydration of the albumen. This step, called plumping, consists of the transfer of water accompanied by a mineral secretion composed of sodium, potassium and bicarbonate. During this phase, the different zones of the albumen become distinguishable: the thick white, the internal and external liquid white, and the chalazes. Their formation results from the slow rotation undergone by the egg in the uterus. This rotation causes a protein twist of the thick white, which like a sponge, releases the zones of liquid white. The shell membranes are stretched under the effect of the pressure exerted by the entry of water. Next, the shell formation occurs by imbrication of calcium carbonate in the form of calcite crystals into the organic matrix. The calcium carbonate is formed from calcium and carbonate ions secreted by the uterus in the uterine fluid.

The secretion of calcium in the uterine fluid occurs without any prior storage of this element in the uterine cells. Calcium and bicarbonate come from the blood and are transferred by the uterus to the uterine fluid that bathes the egg during its formation (Guérin-Dubiard, Anton, Gautron, Nys, & Nau, 2010). The concentration of ionic calcium in the uterine fluid is between 5.5 and 9.5 mM depending on the stage of egg formation. The concentration of bicarbonate, for its part, varies from 90 to 110 mM. In this fluid, the ionic product of the calcium carbonate is more than 60 times greater than the solubility product. This leads to the precipitation of calcium carbonate (Nys, Zawadzki, Gautron, & Mills, 1991).

In the short term, the calcium in the shell comes from the blood. However, the real source of calcium is the diet. The intestine actively participates in the regulation of calcium metabolism. The retention of calcium increases from 40 to 80% when the hen forms

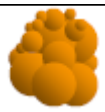
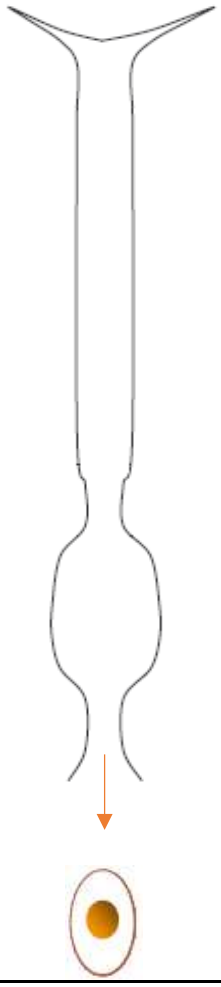
a shell. Some of the calcium deposited on the shell comes from the skeleton. This ability is particularly evident when the diet of the hen is deficient in calcium. The hen can mobilize up to 40% of the calcium of its skeleton. Beyond, the laying ceases (Sauveur, 1988).

1.4.5. Expulsion of the egg

The progression of the egg in the genital tract is made through the contractile activity of its different parts. Shortly before ovulation, the infundibulum is animated by contractions. These contractions place it facing the follicle before ovulating. The passage of the egg into the magnum and the isthmus is ensured by inhibition of the smooth muscles posterior to the egg, coordinated with an important activity of the anterior muscles. The penetration of the egg into the uterus is accompanied by the activation of muscle contractions. The contractile activity of the uterus ensures a slow rotational movement of the egg around its long axis, which lasts throughout shell formation.

After the 2 to 3 hours during which the deposition of the cuticle and the pigmentation are completed, the uterine contractions reach a peak and cause the expulsion of the egg in the vagina, and a few minutes later, outside. Oviposition involves the relaxation of abdominal muscles and sphincter between the shell gland and vagina, and the contraction of smooth muscles of the shell gland (Johnson, 2015).

Table 1.2. Main stages of egg formation (Sauveur, 1988)

Part of the reproductive tract		Length		Functions	Duration
OVARY	Follicles			Vitellogenesis	
				Ovulation	
OVIDUCT	Infundibulum	8 – 11 cm		Fecundation Formation of the perivitelline membrane	15 – 30 min
	Magnum	30 – 35 cm		Formation of the albumen	2 – 3 h
	Isthmus	9 – 15 cm		Formation of shell membranes	1 – 2 h
	Uterus	9 – 11 cm		Deposition of the shell	18 – 20 h
	Vagina	8 – 10 cm		Oviposition	10 min

1.5. Regulation of ovulation and oviposition

Reproduction in the laying hen is regulated according to a circannual rhythm, and this rhythm is phased by an increasing photoperiod, which is detected by photoreceptors in the brain. Unlike mammals, birds have extra-retinal photoreceptors in the pineal organ and deep brain. When the photoperiod increases, long day-induced thyrotropin (TSH) from the pars tuberalis of the pituitary gland causes local activation of thyroid hormones in the mediobasal hypothalamus. In turn, the bioactive thyroid hormone, T3, regulates the seasonal secretion of GnRH, and hence the secretion of gonadotropins (Yoshimura, 2013). Seasonal ovarian growth is stimulated in almost all bird species by increasing the photoperiod. Additional stimuli such as the quality and abundance of food or nesting sites, temperature or interactions with a male, are often required to initiate follicular growth and maturation (Johnson, 2015).

The time from ovulation of an oocyte to the oviposition of the calcified egg generally ranges from 24 to 26 hours and ovulations continue uninterrupted for several days, or up to 1 year or more in extreme cases. The number of eggs laid on successive days is called a sequence, and each sequence is separated by one or more pause days during which no egg is laid. In females in the peak of the sexual season, the pauses last only one day while the sequence consists of one to several dozen ovipositions.

Oviposition in hen is triggered by progesterone and prostaglandins E and F, which are secreted by the periovulatory follicle, in response to preovulatory discharge LH by the pituitary gland. Ovulation usually follows an oviposition within 15 – 75 min except when the hen lays an egg late in the afternoon. In this case, the hen does not ovulate shortly after laying because it is too late in the day and the hen has exceeded the time of its open period for the release of LH. The hen will not lay an egg the next day (pause day). The hen will hold the mature F1 follicle overnight and ovulate it at the very beginning of the next open period for LH release, and a

new sequence will begin. The fact that the hen holds the follicle overnight (about 16 additional hours) causes the first yolks in a sequence to be heavier because the egg yolk still accumulates during this period. Since the size of the yolk affects the size of the egg, the first eggs in the sequence are heavier than subsequent eggs in a sequence (Robinson & Renema, 2003).

1.6. Conclusion

Reproduction in hens is characterized by anatomical and functional peculiarities such as the existence of a single functional ovary and oviduct and the production of hard-shell eggs that contain a yolk-rich oocyte. The main precursors of the yolk constituents are vitellogenin and VLDL, which are synthesized in the liver under oestrogen control. The hepatic origin of the constituents of the yolk has the consequence that the content of some of them may depend on the diet of the hen.

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Chapter 2

Structure, composition and nutritional value of the egg

2.1. Introduction

Unlike mammals, the growth of the embryo in birds occurs inside the egg and the mother does not feed the developing young bird. Therefore, the egg must provide a very secure environment and all the nutrients required for the embryo to grow, as well as allow their metabolic use without the possibility of waste disposal. The diversity of the components of the chicken egg and their perfect balance to ensure the growth of an animal explain the exceptional nutritional quality of the egg for humans. In addition to its essential nutrients, the egg is also remarkable because it contains many molecules involved in the physical (shell and shell membranes) and chemical (antibacterial, antiviral, and antioxidant molecules) protection of the embryo. In this chapter, we first present the structural aspect of egg compounds and their interactions in relation to their respective function. Second, the composition of the eggs is examined, including the egg-contained macro- and micronutrients, the presence of functional substances and the bioavailability of nutrients for humans. Then, we deal with improvement in the nutritive value of eggs, with a particular attention paid to the enrichment in fatty acids.

2.2. Structure of the egg

From inside to outside, the main parts of the egg, shown schematically in Figure 2.1, are observed in the order of their deposition along the reproductive tract of the female bird. These are the yolk or vitellus, the albumen or egg white, the shell membranes and the shell. Due to intensive selection over many years, marketed chicken eggs have on average a relatively constant weight of 60 ± 5 g. The relative proportions of each of the parts may vary considerably depending on various zootechnical factors or the conditions and shelf life of the eggs (Guérin-Dubiard, Anton, Gautron, Nys, & Nau, 2010). Table 2.1 shows the typical range of constituents of the egg. These constituents are considered

relatively stable and are mainly modulated by the proportion of white and yolk in the egg. Their relative shares vary considerably according to the age of the hen and, to a lesser extent, individual features, environmental conditions and feeding practices (Nau, Nys, Yamakawa, & Godbert, 2010; Nys & Sauveur, 2004).

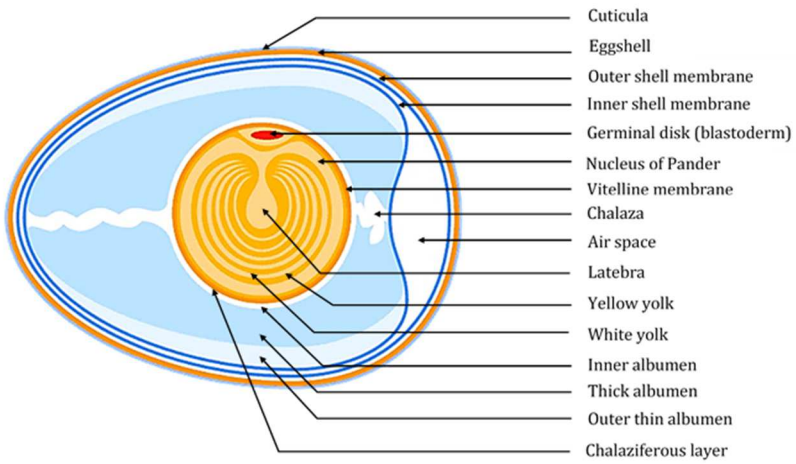

Figure 2.1. Schematic structure of a chicken egg (Sunwoo & Gujral, 2015)

Table 2.1. Proportions of the different parts of the chicken egg (Sauveur, 1988)

	Weight (g)	**% of total egg**	
	Mean	Mean	Extreme values
Shell	5.5	9.1	8.5 – 10.5
Shell membranes	0.25	0.4	–
Albumen	37	61.5	57 – 65
Yolk	17.3	29	25 – 33
Edible parts	54.3	90.5	82 – 98

2.2.1. The egg yolk

The egg yolk is a complex system containing lipid particles dispersed in an aqueous solution of proteins surrounded by a thin, colourless acellular membrane called the vitelline membrane. This protein-made membrane limits exchanges between albumen and yolk and is the ultimate barrier against bacterial penetration (Burley & Vadehra, 1989). Structurally, the yolk consists mainly of «yellow» yolk and «white» yolk. The white yolk represents less than 2% of total egg yolk and comes from the white follicle maturing in the ovary (Yamamoto, Juneja, Hatta, & Kim, 1996). The yellow yolk is an alternation of deep and light layers arranged concentrically (Figure 2.1). The deep yellow yolk is formed during the daytime while the light yellow yolk is formed at night when the protein concentration in the bloodstream is lower than that of the daytime (Wu, 2014).

Morphologically (Figure 2.1), several structures derived from the white yolk are observed in the egg yolk (latebra, neck of latebra, pander nucleus and embryonic disk). Latebra is located in the centre of the egg yolk and is connected to the nucleus of pander through a tube-like wire called neck of latebra. At the top of the clubshaped latebra, on one side of the yolk, is a small disc called germinal disk (2 – 3 mm in diameter). This is the place of the division of the embryonic cells. Once fertilized, the germinal disk divides and grows. It is called blastoderm after it reached the 64-cells stage. The embryo develops only from this small group of cells; the large mass of yolk serves to provide food (Belitz, Grosch, & Schieberle, 2009; Sunwoo & Gujral, 2015; Yamamoto et al., 1996).

The vitelline membrane of approximately 10 µm thick consists of two main layers: the inner layer deposited in the ovary, and the outer layer formed in the oviduct. Both are separated by a thin continuous membrane. The inner and outer layers are three-dimensional mesh structures with fibres of 200 – 600 nm and 15 nm diameter, respectively. While the continuous membrane is a piled, sheet-like structure composed of granules of about 7 nm in

diameter (Mine, 2008; Stadelman, Newkirk, & Newby, 2017; Yamamoto et al., 1996).

2.2.2. The egg albumen

The albumen of the egg is an aqueous, faintly straw-tinted, gel-like liquid (Belitz et al., 2009), consisting of the juxtaposition of four physically distinct zones:

- The chalaziferous layer is a gelatinous layer that closely surrounds the entire egg yolk. In the long axis of the egg, the chalaziferous layer is twisted at both sides of the yolk membrane and forms a thick rope-like structure called chalazae cord (Figure 2.1). The chalazae are filaments going to both extremities of the egg yolk through the thick albumen. They look like two twisted cords, twisted clockwise at the large end of the egg and counterclockwise at the small end (Yamamoto et al., 1996). They maintain the yolk suspended and serve as anchors to keep the yolk in the centre inside the egg.

- The internal liquid white (inner thin albumen) is enclosed between the thick albumen and the chalaziferous layer.

- The thick albumen is attached to the ends of the egg and has the appearance of a gel. In the fresh egg, it covers the inner albumen and chalaziferous layer, keeping the yolk in the centre of the egg. The viscosity of the thick albumen is much higher than that of the thin albumen.

- The external liquid white (outer thin albumen) is in contact with shell membranes and spreads rapidly when the egg is broken on a flat surface.

These different zones of the albumen are distinguished in particular by their water content. The proportion of each varies widely depending on breed, environmental conditions, egg size and

throughout egg conservation after spawning (Guérin-Dubiard et al., 2010; Romanoff & Romanoff, 1949).

2.2.3. The shell membranes

The eggshell membrane structure consists of the inner and outer membranes of 22 and 48 µm thick, respectively (Nys & Gautron, 2007). Both inner and outer membranes are made up of layers of intertwined protein polysaccharide fibres, extending parallel to the surface of the egg, thus limiting the spread of albumen (Nys et al., 2010). The inner shell membrane is in direct contact with the albumen while the outer membrane is situated between the inner membrane and the calcified part of the shell. They strongly adhere to one another except at the level of the air space. This part does not exist at the time of egg-laying but develops immediately by a separation of the two shell membranes at the large end of the egg, when cooling causes the contents of the egg to shrink (Sunwoo & Gujral, 2015).

2.2.4. The eggshell

The eggshell is a protective mineralized envelope, which limits the contamination of the egg by the germs and allows, thanks to its porosity, gas exchanges between the external environment and the embryo. Indeed, several funnel-shaped pore canals are distributed unevenly on the shell surface. Their number varies from 7,000 to 15,000 with a density of 70 to 200/cm^2, mostly numerous at the broad end of the egg where the air space appears (Sauveur, 1988). From inside to outside, the shell structure is divided into four layers (Figure 2.2):

– The cone or mammillary layer (inner calcified layer) of about 70 µm thick is composed of the basal parts of calcified columns and cones that penetrate the outer shell membrane. Some organic cores called mammillary knobs are deposited on the

surface of the outer eggshell membrane during the passage of the forming egg in the isthmus. These mammillary knobs are the sites of mineralisation on which the calcium carbonate crystals deposition is initiated.

– The palisade layer (200 µm thick) represents two-thirds of the total thickness of the shell. It starts where the multi-directional growth of the cones of the mammillary layer causes the adjacent cones to cement. This layer consists of a compact layer of calcite crystals associated with an organic matrix. This continuity is broken at the level of the pores that pass through the shell, to allow gaseous exchanges between outside and inside.

– The vertical crystal layer is located at the upper part of the palisade layer. It's a narrow monocrystalline band of vertically oriented crystals that are perpendicular to the shell surface (Nys & Gautron, 2007).

– The cuticle is the outmost shell coating. It consists of an extremely thin protein layer (10 µm), transparent and mucilaginous. It covers the entire surface of the egg and partially seals the pores, thus restricting microorganism penetration while the permeability to gases is remained (Belitz et al., 2009). The gaseous exchanges are made possible by cracks appearing in the cuticle in the first minutes after egg-laying (Guérin-Dubiard et al., 2010).

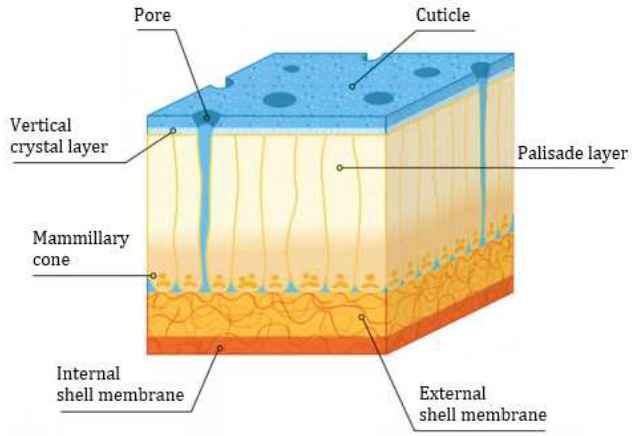

Figure 2.2. Schematic structure of chicken eggshell, including shell membranes (Hincke et al., 2012)

2.3. Composition of the egg

The overall composition of the egg per 100 g of fresh consumable matter is reported in Table 2.2. The egg is composed of 61% white (saline containing ~ 11% protein) and 29% yolk (~ 51% water, ~ 16% protein, ~ 31% lipid), contained in a shell that represents 10% of the total weight. The edible part of the egg contains on average 75% water, while proteins (12%) and lipids (10 – 11%) are the main contributors to the nutritional value (Guérin-Dubiard et al., 2010). Small amounts of carbohydrate and minerals or ashes are also present. Proteins are present mainly in albumen and yolk, while lipids are almost exclusively in yolk. Minerals are the main component of the shell (Mine, 2008).

Table 2.2. Average composition of chicken egg (adapted from Guérin-Dubiard et al., 2010; Mine, 2008 and Nys & Sauveur, 2004)

	In g per 100 g of each part			
	White	Yolk	Eggshell	Whole*
Water	88	51.1	1.6	75.6
Proteins	10.6	16	6.2	12.8
Lipids	-	30.6	0.03	10.5
Carbohydrates	0.9	0.6	Trace	0.3
Ashes	0.5	1.7	92	0.8

(*) Relative to the whole egg without shell

2.3.1. Composition of shell and shell membranes

The avian eggshell is a natural porous bioceramic of about 0.3 mm thick. With shell membranes, it contains on average 1.6% of water, 3.3% of organic material and 95.1% inorganic material. The organic material is present in all layers of the shell. However, its content varies between different parts. The shell membranes and the cuticle are made up exclusively of an organic matrix. The shell without the cuticle is composed of a small proportion of organic constituents (about 2.5% of the calcified layer) (Nys & Gautron, 2007; Nys et al., 2010). The organic matrix of the shell is a complex mixture of proteins and polysaccharides. The proteins of the organic matrix can be classified into three groups:

– Egg white proteins, in particular ovalbumin, ovotransferrin and lysozyme that are also present in the eggshell

– Ubiquitous proteins including osteopontin, a phosphorylated glycoprotein found in bones, kidneys and other hard tissues of birds and mammals, and clusterin, a widely distributed secretory glycoprotein

– Eggshell-specific matrix proteins, unique to the calcification process of the shell, which are exclusively secreted by

cells of the red isthmus and uterus, these components being called ovocleidins and ovocalyxins, and being identified, with few exceptions, only in the domestic hen (Nys & Gautron, 2007; Nys, Hincke, Arias, Garcia-Ruiz, & Solomon, 1999; Nys, Gautron, McKee, Garcia-Ruiz, & Hincke, 2001).

In contrast to the organic matrix, the mineral phase is not present in all layers of the shell. The eggshell mineral consists mostly of calcium carbonate in the form of calcite, which accounts for 98.4% of the mineralized part. The remaining 1.6% consist of magnesium carbonate and calcium phosphate, the latter being concentrated in the upper layers. The mineral phase also contains many oligo-elements including manganese (7 ppm) whose feed intake in the hen favours the strength of the shell by probably influencing its crystalline structure (Nys et al., 2010). The cuticle on the surface of the shell consists of 3% minerals, 5% sugars and more than 90% proteins and glycoproteins. It contains a layer of hydroxyapatite and, in the case of colored shells, nearly two-thirds of brown pigments (protoporphyrins) (Nys, Zawadzki, Gautron, & Mills, 1991).

The shell membranes are composed of collagen-like proteins (collagen type I, V and X) and other fibrillar proteins containing lysine-derived cross-links (Nys et al., 1999; Sunwoo & Gujral, 2015). On the surface of the outer membrane of the shell, are mammillary knobs that contain proteoglycans among which keratan sulphates (Guérin-Dubiard et al., 2010; Nys & Gautron, 2007).

2.3.2. Egg white components

The egg white is mainly composed of water and proteins. Table 2.3 shows the proportions and moisture content of the various layers of the albumen. The moisture decreases from the outer to the inner albumen.

Table 2.3. Proportion and moisture content of the different layers of egg white (Guérin-Dubiard et al., 2010; Romanoff & Romanoff, 1949)

Layers	**% Albumen**		**% Moisture**
	Mean	Range	
Outer thin albumen	23.2	10 – 60	88.8
Thick white	57.3	30 – 80	87.6
Inner thin albumen	16.8	1 – 40	86.4
Chalazes	2.7	–	84.3

2.3.2.1. Proteins of albumen

Proteins are the major component of the egg white dry matter and make up 11% of its entire composition. The egg white proteins are for the most part glycoproteins. In fact, the albumen can be considered as an aqueous solution of globular proteins in which bath glycoprotein fibres, particularly abundant in the thick white (Guérin-Dubiard et al., 2010). Egg white proteins, because they come from the *de novo* synthesis of proteins in the magnum, have a great stability in their content and composition (Nys & Sauveur, 2004). The main proteins are ovalbumin (54%), ovotransferrin (12 – 13%), ovomucoid (11%), lysozyme (11%) and ovomucin (1.5 – 3.5%). The protein compositions of the thin and thick albumen layers differ particularly in their ovomucin content. The ovomucin content in thick white is approximately four times that of the thin white (Sunwoo & Gujral, 2015).

Because of their nutritional, techno-functional and biological properties, egg white proteins have been widely studied. However, they are not yet fully characterized. For the time, there are more than 12 different protein families. Besides the fact that some of them exist in very large quantities, such as ovalbumin, other proteins are found in egg white in trace amounts. Their molecular weights are highly variable (12.7 – 8000 kDa) and, with the exception of lysozyme and avidin (0.05% of albumen proteins), they have an acidic isoelectric point (pI) (Guérin-Dubiard et al., 2010). Several albumen proteins, in addition to providing amino acids to the developing chick, have biological activities mainly related to the protection of the egg against microbial deterioration (e.g. ovalbumin, ovotransferrin, ovomucoid, lysozyme, ovomucin, avidin, etc). Other activities include providing the egg white with valuable functional properties such as viscosity, thermogelling, foaming and emulsification (e.g. ovomucin).

2.3.2.2. Carbohydrates of albumen

Carbohydrates constitute 0.9% of the albumen, existing both in free form and in combination with proteins. Glucose is the main free sugar (~ 50%) and the others, such as galactose and mannose, are present as N- or O-linked oligosaccharides conjugated to albumen proteins (Guérin-Dubiard et al., 2010; Mine, 2008).

2.3.2.3. Minerals of albumen

The elemental composition of the egg white (Table 2.4) is extremely variable. The mineral content depends on many factors, including zootechnical parameters. The mineral content of the hen's diet is the most important factor influencing the amount of specific minerals in albumen (Romanoff & Romanoff, 1949; Stadelman et al., 2017). Thus, it is possible to enrich the content of the egg in trace elements such as iodine, selenium, fluorine or

manganese. On the other hand, copper and zinc contents are relatively more stable because these elements are associated with proteins before being transferred to the egg (Nys & Sauveur, 2004).

2.3.2.4. Vitamins of albumen

The albumen is devoid of fat-soluble vitamins, but contains significant proportions of group B vitamins including biotin, niacin and riboflavin, which are water-soluble vitamins. However, the albumen and the egg in general lack vitamin C. Many vitamins are in bound form with proteins. Table 2.4 reports the average vitamin composition of the egg.

2.3.3. Major constituents of egg yolk

The yolk makes up about 30% of the weight of the whole egg. Its main constituents are lipids and proteins in a 2:1 ratio. The yolk is a fat-in-water emulsion with about 50% dry matter content and consists of about 51.1% water, 30.6% lipids, 16% proteins, 1.7% minerals and 0.6% carbohydrates. The composition of the vitelline membrane differs from that of the yolk. Its solid matter is higher in proteins (87%) and carbohydrates (10%) than in lipids (3%) (Anton, 2007a; Li-Chan & Kim; Robinson, Fasenko, & Renema, 2003).

2.3.3.1. Egg yolk lipids

The lipids in egg yolk are made up of two thirds of triglycerides (~ 65%), but also include phospholipids (~ 29%), cholesterol (~ 5%) and free fatty acids (~ 1%). Carotenoids represent less than 0.1%, and give the yolk its colour. Yolk lipids are integrally associated with proteins. These interactions between lipids and proteins result in the formation of lipoprotein complexes. Thus, lipids are exclusively distributed in low-density

lipoproteins (LDL) and high-density lipoproteins (HDL) (Guérin-Dubiard et al., 2010).

Table 2.4. Average vitamins composition of the egg (adapted from Guérin-Dubiard et al., 2010 and Nys & Sauveur, 2004)

Micronutrients	**Whole** (shell excluded)	**White**	**Yolk**
Mineral (mg/100 g fresh weight)			
Sodium (Na)	120	155	50
Chlorine (Cl)	172	175	162
Calcium (Ca)	50	8	133
Phosphorus (P)	193	18	530
Magnesium (Mg)	12	10	15
Iron (Fe)	1.7	0.1	4.8
Zinc (Zn)	1.3	0.12	3.9
Copper (Cu)	0.06	0.02	0.14
Manganese (Mn)	0.04	0.007	0.11
Iodine (I)	0.05	0.003	0.14
Sulphur (S)	164	163	165
Vitamins (µg/100 g fresh weight)			
Water-soluble vitamins			
Ascorbic acid (C)	0	0	0
Thiamine (B_1)	91	10	250
Riboflavin (B_2)	447	430	480
Niacin (B_3)	79	90	60
Pantothenic acid (B_5)	1700	250	4500
Pyridoxine (B_6)	138	10	370
Biotin (B_8)	25	7	60
Folic acid (B_9)	60	12	140
Cobalamin (B_{12})	1	0.1	2.8
Fat-soluble vitamins			
Retinol equivalent (A)	150	0	450
Cholecalciferol (D_3)	1.5	0	4.5
α-tocopherol (E)	1300	0	3600
Vitamin K	4.25	0	15

The fatty acid composition of lipids, based on a standardized feed of hens, is approximately 30 – 35% saturated fatty acids (SFA), 40 – 45 % monounsaturated fatty acids (MUFA), and 20 – 25% polyunsaturated fatty acids (PUFA). The main representatives of SFA and MUFA are palmitic acid (C16:0) and oleic acid (C18:1n-9), respectively, whereas the major PUFA is linoleic acid (C18:2n-6). Alpha-linolenic acid (C18:3n-3) is a minor component in eggs from hens fed with common commercial diets (Kuksis, 1992; Speake, Murray, & Noble, 1998). This composition can vary considerably depending on the dietary fatty acids ingested by the hen (Bouvarel, Nys, Panheleux, & Lescoat, 2010; Guérin-Dubiard et al., 2010).

Egg yolk phospholipids are very rich in phosphatidylcholine (PC), which accounts for 76% of total phospholipids. Phosphatidylethanolamine (PE) represents 20% of the phospholipids, while phosphatidylinositol (0.7%), phosphatidylserine (0.9%), sphingomyelin (0.5%), cardiolipins (0.3%), and lysoPC and lysoPE (1%) are present in very low amounts (Kuksis, 1992).

Avian eggs contain large amounts of cholesterol that are found exclusively in the yolk. Cholesterol is the essential sterol of egg yolk. It comes from the feeding of hens and from the synthesis in the liver during the elaboration of lipoproteins. Cholesterol accounts for about 5% of total lipids. While most of the cholesterol exists as free cholesterol (85 – 90%), a minor proportion is present as cholesteryl ester (10 – 15%) (Bitman & Wood, 1980). Free cholesterol enters into the structure of LDL, while cholesterol esters are present in the lipid core of LDL (Kuksis, 1992).

Carotenoids give hen's egg yolk its yellow colour, which can range from a very pale yellow to a dark brilliant orange. These pigments are economically important because colour is a quality criterion for the consumer acceptability of commercial eggs. Carotenoids in yolk are mainly carotene and xanthophylls (lutein, cryptoxanthin and zeaxanthin). The yolk pigments are transported through the blood by the lipoproteins, which are deposited in the

yolk (Sunwoo & Gujral, 2015). Pigments cannot be synthesized by the metabolism of the hen. Therefore, they come from the food (Anton, 2007a).

2.3.3.2. Egg yolk proteins

In yolk, the majority of proteins is found in the form of lipoproteins consisting of LDL and HDL (68 and 16% of total proteins, respectively). Free proteins are made of globular proteins (livetins), phosphoproteins (phosvitin), and minor proteins (10, 4 and 2% of total proteins, respectively) (Anton, 2007a). The yolk can be separated by dilution and mild centrifugation into two distinct fractions: plasma and granules (Figure 2.3). Plasma represents 77 to 81% of the yolk dry matter and consists of 85% of LDL and 15% of livetins. Granules represent about 22% of the dry matter of the yolk, mainly constituted by HDL (70%) and phosvitin (16%). LDL (12%) called LDLg are also included in the granules structure (Anton, 2013). The main proteins identified in the HDL and LDL structures of plasma and granules are listed in Table 2.5.

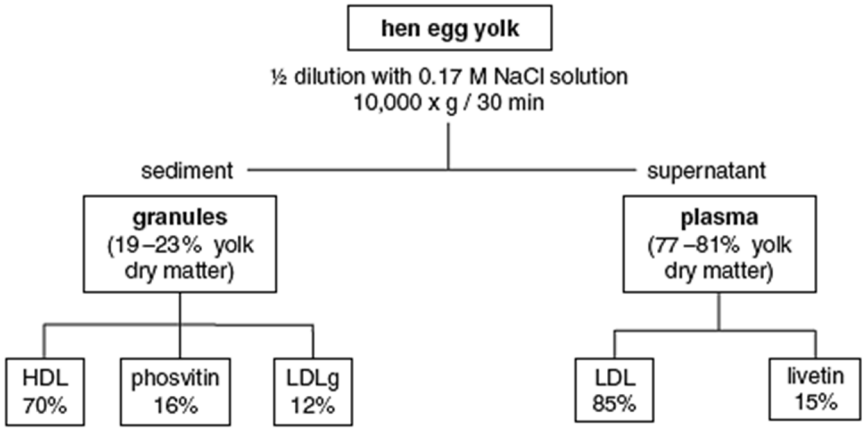

Figure 2.3. Diagram of the fractionation of egg yolk into granules and plasma (Anton, 2007a)

i. Low-density Lipoproteins (LDL)

LDL are the main components of yolk. They represent about two-thirds of the total yolk dry matter, and are thought to be responsible for the functional properties of yolk, in particular its emulsifying capacity (Li-Chan & Kim, 2008). LDL are primarily located in plasma, but a small portion is included in granules (LDLg). They are micellar structures (17 – 60 nm) with a neutral lipid core of triglycerides and cholesterol esters surrounded by phospholipids and proteins, called apoproteins. Free cholesterol is included in the phospholipid film, increasing its rigidity (Figure 2.4). LDL are composed of 11 to 17% of proteins and 83 to 89% of lipids, of which 74% are neutral lipids and 26% phospholipids (Anton, 2007c).

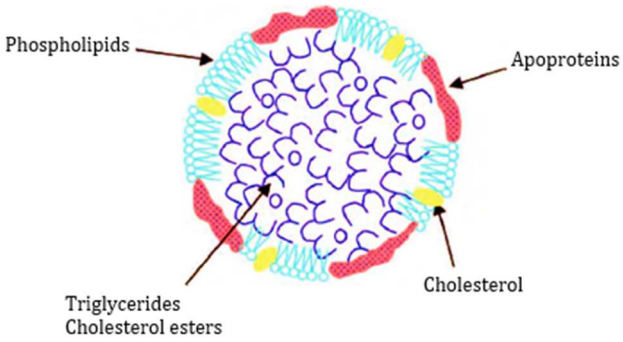

Figure 2.4. Schematic representation of LDL of egg yolk (Anton, 2007c)

The VLDLy synthesized in the liver of the hen are the precursors of egg yolk LDL. They are transported by the blood to the ovary, where they are transferred by receptor-mediated endocytosis into the oocyte. There are six apoproteins of LDL. VLDLy mainly contain apoVLDL-II and apoB, both of which make up over 90% of their proteins. Apo VLDL-II is a small protein containing 82 amino acid residues and accounts for 40% of the VLDLy proteins. ApoB is a 500 kDa protein consisting of a single

subunit, highly similar but with higher molecular weight than the mammalian apoB-100 (Anton, 2007c; Williams, Wang, & Capony, 1979). When VLDLy are transferred into the yolk, apoB is cleaved enzymatically, resulting in the production of apoB fragments. ApoVLDL-II, called apovitellenin I in yolk, is the only apoprotein from blood lipoproteins to be transferred in large amount to yolk without modification. Apovitellenin I is a small homodimer with disulfide-related subunits of 9.5 kDa. There is a lack of knowledge about the identification of other LDL apoproteins, and concerning the exact maturation mechanism of apoprotein precursors (Anton, 2007c).

Table 2.5. Main proteins involved in the HDL and LDL structures of plasma and granules in the egg yolk (Guérin-Dubiard et al., 2010)

Proteins	**Nature**	**Structural components**	**Localisation**
Apovitellenin I	Lipoprotein	LDL	Plasma
Apolipoprotein B *(apo B)*	Lipoprotein	LDL	Plasma
α-Lipovitellin	Lipoprotein	HDL	Granules
β-Lipovitellin	Lipoprotein	HDL	Granules
α-Livetin	Protein	-	Plasma
β-Livetin *(α_2-glycoprotein)*	Protein	-	Plasma
γ-Livetin *(γ-globulin)*	Protein	-	Plasma
Phosvitin	Protein	HDL-phosvitin complex	Granules

ii. High-density Lipoproteins (HDL)

HDL are specifically located in the granules and are called lipovitellins. HDL have a diameter from 7 – 20 nm and a molecular weight of about 400 kDa. They are in the form of a dimer of two monomers, α- and β-lipovitellins, of about 200 kDa each. Both lipovitellins have been shown to be glycoproteins containing mannose, galactose, glucosamine and sialic acid. Lipovitellins are made up of 75 – 80% of proteins and 20 – 25% of lipids. HDL do not have the spherical micelle-like structure of LDL, but rather a pseudo-molecular structure similar to that of globular proteins. They are not formally lipoproteins, more closely resembling lipid transfer proteins.

Lipovitellins result from the proteolytic cleavage of vitellogenin, synthesized under oestrogenic regulation in the liver, where it is phosphorylated and glycosylated (Anton, 2007b). Vitellogenin consists of α- and β-lipovitellins and phosvitin, which are the main phosphoproteins in egg yolk. Vitellogenin is not present in the immature hen. Its synthesis is highly stimulated just before the start of egg production, induced by oestrogens. Vitellogenin undergoes post-translational modifications before its secretion in plasma by phosphorylation on serine residues that are largely present in phosvitin, glycosylation of phosvitin and lipidation of lipovitellins. It also binds to calcium through sites present in phosvitin. Vitellogenin is dissociated into lipovitellins and phosvitin by proteolytic cleavage mediated by cathepsin D, after its transfer to the yolk. Cathepsin D is synthesized by the oocyte and stored in specialized organelles located at the surface of the egg where yolk precursors are integrated (Nys & Guyot, 2011). Homologous sequences have been identified on apoB and vitellogenins. Therefore, they could use the same membrane receptor for endocytosis (Banaszak, Timmins, & Poliks, 1990).

iii. Phosvitin

Phosvitin is a phosphoglycoprotein that represents 4% of the yolk dry matter. It is the second constituent of granules after HDL and represents 16% of the mass of granules. Like HDL, it is one of the proteolytic cleavage products of vitellogenins (Anton, Castellani, & Guérin-Dubiard, 2007). Of the yolk proteins, phosvitin is the most phosphorylated; almost 50% of its amino acids are serine, 90% of which are phosphorylated. Phosvitin consists of two polypeptides: α-phosvitin (160 kDa) and β-phosvitin (190 kDa). These polypeptides contain 3% (α) or 10% (β) phosphorus that represent approximately 80% of the total protein phosphorus of yolk. Unlike most other yolk proteins, phosvitin is not associated to lipids (Anton et al., 2007; Joubert & Cook, 1958).

iv. Livetin

Livetins are non-lipid associated globular proteins present in the plasma. They represent 11% of the yolk dry matter and 30% of the plasma proteins. The livetins are blood proteins deposited in the yolk. They are composed of α-livetin, β-livetin, and γ-livetin with average molecular weights of 80 kDa, 45 kDa and 170 kDa, respectively (Schade & Chacana, 2007).

The α-livetin of egg yolk is identical to the serum albumin of the hen. It is also known as allergen Gal d5, approved by the WHO/IUS Allergen Nomenclature Subcommittee. Alpha-livetin, as well as transferrin and egg white lysozyme, have been implicated as allergens responsible for the bird-egg syndrome, a form of type I hypersensibility with respiratory symptoms (asthma and/or rhinoconjunctivitis). It occurs upon sensitization via egg intake or other contact with bird allergens (feathers, meat…). Bird-egg syndrome is characterized by the development of respiratory and gastrointestinal symptoms. The α-livetin is a partially heat-labile protein. The reactivity of IgE to chicken serum albumin heated for 30 min at 90°C is reduced by 88%. This is why patients with bird-

egg syndrome can tolerate the ingestion of well-cooked eggs and poultry meat (Quirce et al., 2001).

Beta-livetin has been identified as a α-2-glycoprotein. Little information is available on this protein and existing data are ambiguous (Schade & Chacana, 2007).

Egg yolk γ-livetin or γ-globulin is referred to as yolk immunoglobulin (IgY) to distinguish it from mammalian IgG (Kovacs-Nolan & Mine, 2004).

v. Minor yolk proteins

Some proteins are present in egg yolk at low concentrations. Some are specifically induced at sexual maturity, such as proteins binding biotin, thiamine, riboflavin, vitamin A and vitamin D (Nys & Guyot, 2011).

2.3.3.3. Egg yolk carbohydrates

Carbohydrates account for less than 1% of the egg yolk content, with 0.3% as free carbohydrate, mainly glucose, and the remainder represented mainly by sialic acid bound to glycoproteins and glycolipids (Li-Chan & Kim, 2008).

2.3.3.4. Egg yolk minerals and vitamins

Minerals represent less than 2% of the egg yolk content. The main mineral is phosphorus, present mainly in bound form in phospholipids. Other minerals are calcium, chloride, potassium, sodium, sulphur, magnesium and manganese (Table 2.4). The diet of hens can be modified to alter the composition of egg yolk minerals.

Most of the vitamins found in eggs are found in the egg yolk rather than in the albumen. Fat-soluble vitamins A, D, E and K and

water-soluble vitamin B_{12} are found exclusively in egg yolk. Other water-soluble vitamins, such as folic acid, riboflavin and niacin, are present in both egg yolk and albumen. Supplementing the diets of hens makes it possible to produce eggs enriched with vitamins, especially vitamin E.

2.4. Nutritional quality and enrichment of the egg

2.4.1. Nutritional quality of proteins

The quality of a protein is mainly related to its essential amino acid content and digestibility. The high content of bioavailable proteins in eggs is of great benefit to human nutrition. As shown in Table 2.6, chicken eggs have a high nutritional quality among foodstuffs, providing all essential amino acids in quantities adapted to human needs, in particular thanks to a high content in lysine and sulphur amino acids. The proteins in raw egg white are poorly digestible (51%) due to the antitrypsic activity of ovomucoid. This activity is destroyed during cooking, which in addition allows, by causing the coagulation of proteins, a stimulation of gastric and pancreatic secretions. The protein digestibility of the cooked egg is thus almost total (91 – 98%). Compared to all animal and vegetable proteins, cooked egg proteins have a very high digestibility (Nys & Sauveur, 2004). However, the digestibility of proteins does not always reflect the digestibility or bioavailability of essential amino acids. The digestible indispensable amino acid score (DIAAS) is intended to predict the quality of dietary proteins in terms of the digestibility of their essential amino acids. This digestibility is based on the true ileal digestibility of each amino acid (i.e. the measurement of amino acid disappearance between the mouth and the end of the small intestine) preferably determined in humans, but if this is not possible, in the growing pig or the growing rat, in that order. Of note, the amino acid digestibility is assumed to be equivalent to crude protein digestibility when amino acid digestibility data are

not available. The score corrected by the digestibility is calculated for each dietary indispensable amino acid. The lowest value is designated as the DIAAS and used as an indicator of the quality of the dietary protein (FAO, 2013). The value of DIAAS is very high for eggs (113%), higher than that of rice, wheat, peas and soybeans but slightly lower than that of milk (Table 2.6).

Some proteins in eggs have been discovered to have various biological activities and are referred to as bioactive proteins. For example, lysozyme, which is found in egg white, has antibiotic properties and can be used to fight infections. Egg yolk immunoglobulin antibodies also exhibit antibiotic characteristics. In addition, they are able to provide passive immunity against many intestinal pathogens in humans and animals (Huopalahti, López-Fandiño, Anton, & Schade, 2007). However, the egg is one of the main allergenic foods of animal origin. It is the main cause of food allergy in children, with 35 to 50% of cases of allergies compared to 7% in adults (Guérin-Dubiard et al., 2010; Rancé & Dutau, 2010). Nevertheless, the prevalence of allergy decreases with age. Allergic manifestations to the egg are usually cutaneous (urticaria or eczema), digestive (vomiting or diarrhoea) or respiratory (asthma). Anaphylactic shock after egg ingestion would be rare (Guérin-Dubiard et al., 2010; Nys & Sauveur, 2004). Four egg white proteins are recognized as the major allergens of the egg: ovomucoid, ovalbumin, ovotransferrin and lysozyme. Ovomucoid has high allergenic reactivity compared to other egg white proteins. It is relatively stable in heat. On the other hand, under reducing conditions, the digestibility of the ovomucoid is improved and its allergenicity reduced. However, peptides derived from the digestion of ovomucoid retain allergenic activity. Ovalbumin is the most abundant protein found in egg white, it is somewhat sensitive to thermal denaturation, thus causing a resultant decrease in allergenicity. Unlike ovomucoid and ovalbumin, ovotransferrin and lysozyme are totally denatured by heat treatment (Mine & Rupa, 2004; Shin, Han, & Ahn, 2013). The heating operations in most

cases lead to changes in the structure of the proteins but do not eliminate the allergenic potential of all proteins. As a result, some people cannot consume eggs in any form while others tolerate cooked eggs but react to raw eggs. Immunotherapeutic approaches have been developed as a strategy against egg allergy. Technological solutions have also been explored such as the development of hypoallergenic products after elimination of the ovomucoid. However, this type of approach remains limited because of the undesirable effects of such treatments on the techno-functional properties of the egg (Guérin-Dubiard et al., 2010).

Table 2.6. Amino acid composition and digestibility of various food proteins (adapted from Burd, Beals, Martinez, Salvador, & Skinner, 2019; FAO, 2013; Guérin-Dubiard et al., 2010 and Ren, Wu, & Renema, 2010)

	Egg	**Rice**	**Wheat**	**Peas**	**Soy**	**Beef**	**Cow milk**	**Requirements (adult)**
Protein content (%)	12.8	6.7	12.2	22.5	38	17.7	3.5	
				mg/g protein				
Histidine	23	25	25	23	28	34	34	16
Isoleucine	53	44	35	43	50	48	63	30
Leucine	84	87	71	68	85	81	123	61
Lysine	66	38	31	75	70	89	71	48
Methionine + cysteine	52	39	43	20	28	40	33	23
Phenylalanine + tyrosine	93	85	80	73	88	80	131	41
Threonine	48	35	31	41	42	46	44	25
Tryptophan	15	–	–	–	14	–	13	7
Valine	64	61	47	47	53	50	73	40
Digestibility (%)	98	75	91	88	95	98	95	
DIAAS (%)*	113	60	45	65	89	112	116	

Digestibility = 100 x [(amount of dietary amino acid ingested – amount of dietary amino acid recovered from the digesta in the ileum) / amount of dietary amino acid ingested]

DIAAS (Digestible indispensable amino acid score) = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1g of the reference protein)]

* DIAAS were calculated using true ileal indispensable amino acid digestibility values and reference amino acid pattern for children older than 3 years, adolescents, and adults

2.4.2. Lipid metabolism in hen and nutritional characteristics of egg lipids

Most of the lipids in the yolk are found in the LDL. This fraction results from the transfer into the yolk of almost unmodified hen-specific lipoproteins (VLDLy), transported through the blood plasma after their synthesis in the liver. Upon absorption through the intestinal lumen, hydrolysed products of lipid digestion, consisting of long-chain fatty acids and monoacylglycerols, are re-esterified in the endoplasmic reticulum of enterocytes prior to transport. The resulting triglycerides are packed with cholesterol, phospholipids, and proteins to form lipoproteins. Since the intestinal lymphatic system is poorly developed in hens, lipoproteins are secreted directly into the portal system and are therefore called portomicrons (Bensadoun & Rothfeld, 1972). Their size (about 150 nm in diameter) and composition are very similar to those of mammalian chylomicrons (Griffin, Grant, & Perry, 1982). Portomicrons pass through the liver before reaching the rest of the circulation. On the other hand, short-chain fatty acids and free glycerol are transported as such to the liver, also via the portal system. In the liver, extensive reprocessing of glycerides and fatty acid residues from the portomicrons occurs. The lipids reform and are included in VLDL particles, which then pass to the Golgi complex where they acquire phospholipids and further glycosylation. Finally, the complete particles of VLDL concentrate in secretory vesicles, then flow into the blood (Yannakopoulos, 2007).

In laying hens, lipoprotein metabolism is adapted, on one hand, to facilitate massive transport of nutrients into the growing oocytes and, on the other, to maintain systemic lipid homoeostasis (Figure 2.5). VLDLy particles, whose synthesis is massively elevated by oestrogen at the onset of spawning, function primarily as lipid carriers from the liver to the oocytes. VLDLy have a standard structure consisting of a core of triglycerides and cholesterol esters surrounded by a surface layer composed of

phospholipids, free cholesterol and apoproteins. Their size is reduced to a diameter of about 30 nm. These features facilitate the transfer of VLDLy into the ovarian follicular wall. Other important particularities of VLDLy are the presence of apoVLDL-II present only in egg-producing hens, and apoB to the detriment of other apolipoproteins (apo-AI, C-type derived apolipoproteins). The catabolism of VLDLy is limited before their transfer into the yolk, so that they remain similar to their native hepatic form. However, just before entering the oocyte within the follicle, VLDLy pass through the ovarian granulosa cell monolayer, a major site of lipoprotein lipase (LPL) expression. ApoVLDL-II plays an important role in preserving VLDLy particles by inhibiting LPL. Oocyte endocytosis of VLDL is mediated by an LR8 receptor that recognizes the apoB moiety of VLDL and vitellogenin (Nimpf & Schneider, 1998; Nys & Guyot, 2011).

Egg lipids are mainly in the form of triglycerides, phospholipids, and free cholesterol. The minor components of yolk lipids include cholesterol esters and free fatty acids. The triglyceride component exhibits high levels of unsaturated fatty acids. The phospholipid fraction consists of almost 80% phosphatidylcholine. Egg phospholipids are a good dietary source of choline, and choline is important for the synthesis of phospholipids in cell membranes, neurotransmission, transmembrane signalling, and lipid-cholesterol transport and metabolism (Ren et al., 2010). In addition, the digestibility of egg triglycerides is excellent (98%), and that of phospholipids is also satisfactory (90%) (Nys & Sauveur, 2004).

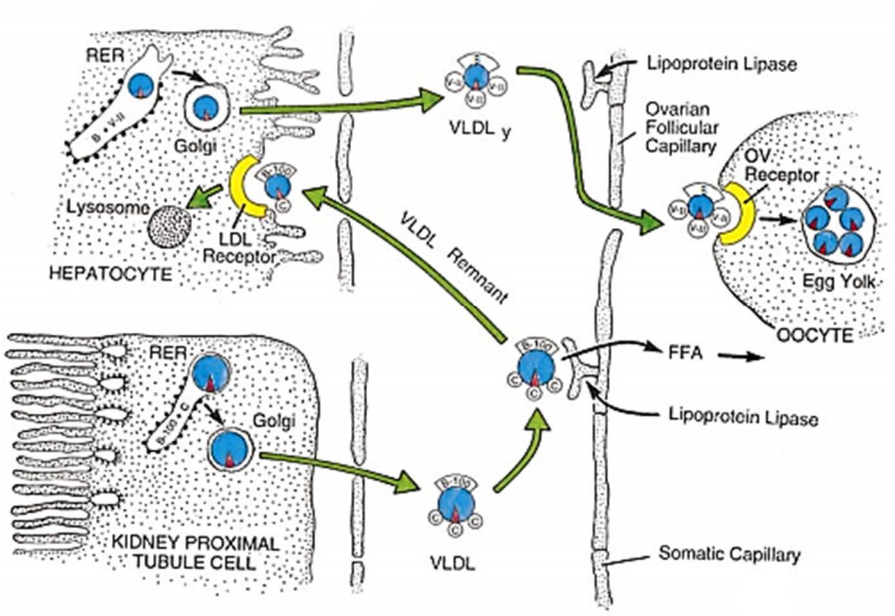

Figure 2.5. VLDLy pathway from hepatocyte to egg yolk. Egg-laying hen hepatocytes (upper left) synthesize yolk-targeted very low density lipoproteins (VLDLy) coated with apolipoprotein B and apolipoproteinVLDL-II that blocks lipoprotein lipase hydrolysis (upper right). VLDLy particles that are small diameter (~ 30 nm) pass readily through ovarian capillaries where they are taken up intact by receptor-mediated endocytosis in oocytes and comprise the bulk of the egg yolk. Kidney proximal tubule cells (lower left) assemble generic-sized VLDL (~ 60 nm) that interact with somatic capillary lipoprotein lipase (lower right), providing free fatty acids (FFA) to tissues such as cardiac muscle, skeletal muscle and adipose tissue (Walzem et al., 1999).

The lipid composition of the yolk is the result of a combination of hepatic *de novo* lipogenesis and incorporation of lipid components from the diet of the hen. Thus, the egg yolk can be enriched with specific fatty acids by incorporating fats rich in these fatty acids in the feed intended for hens. Eggs contain a high proportion of n-6 PUFA, but are generally a poor source of n-3 fatty acids. There are two main approaches to increase the n-3 PUFA content of eggs, either by enriching the hen's diet with α-linolenic

acid (ALA) from a plant source (flaxseed, canola, etc.), or by adding any source of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) provided by fish oil or marine algae. Flaxseed, owing to its high fat (~40 – 42%) and ALA (> 50%) contents along with other nutritional properties (e.g., metabolizable energy, protein content), is the most widely used ingredient in the production of eggs rich in n-3 PUFA. Several authors reported successful enrichment of eggs with n-3 PUFA by incorporating flax in the hens' diets (Bean & Leeson, 2003; Caston & Leeson, 1990; Cherian & Hayat, 2009). Overall, the greatest effect of feeding flax is the increase in ALA in eggs. Studies reported an increase from 100 to 300 mg ALA in eggs from hens fed 5 to 15% flaxseed (by weight of feed). The dramatic increase observed in ALA was not associated with a comparable increase in long-chain n-3 PUFA. However, flaxseed feeding may result in an increase of the DHA content of eggs, from about 20 to about 100 mg/egg. More generally, it leads to a reduction in n-6 PUFA and n-6/n-3 PUFA ratio. The effect of flaxseed feeding is limited on saturated fatty acids, but several studies reported a decrease in oleic acid (Beheshti Moghadam & Cherian, 2017; Cherian, 2016).

Menhaden and herring oils contain about 1% ALA but a total of 12 – 14% EPA and DHA combined. The use of fish oils has the advantage of enriching the eggs mainly with long-chain n-3 PUFA. Adding 3% menhaden oil in the hens' diet could increase EPA up to 30 mg/egg and DHA to over 190 mg/egg (González-Esquerra & Leeson, 2001). The maximum level of fish oils that can be used in the diet of layers is limited due to the production of fish flavoured eggs. It seems that treatments with less than 3% oil do not have a significant negative effect on the quality of the flavour (Van Elswyk, Dawson, & Sams, 1995). Another approach to enriching eggs with long-chain n-3 PUFA is to use microalgae in the diet of laying hens. Different types of microalgae can contain up to 50% of their total fatty acids as EPA and DHA (Yannakopoulos, 2007). The possibility of modifying the fatty acid composition of the egg yolk led also to

the development of eggs enriched with particular fatty acids such as conjugated fatty acids. This topic will be discussed in the next chapter.

Eggs are one of the richest sources of cholesterol in the human diet. One large egg yolk contains approximately 200 mg of cholesterol. Cholesterol is a structural component of cell membranes and a precursor of hormones, vitamin D and bile acids. The amount of cholesterol in eggs and its contribution to total human cholesterol intake is still a contentious issue with the suspicion of a role of the dietary cholesterol in the development of cardiovascular diseases (CVD). Large-scale epidemiological studies have found only weak associations between egg consumption and the risk of CVD. Clinical studies showed that the impact of dietary cholesterol via egg intake on serum lipids is highly variable, with the majority of individuals (~ 2/3 of the population) having minimal responses, while those with a significant response increase both LDL and HDL-cholesterol, usually with maintenance of the LDL/HDL-cholesterol ratio (Blesso & Fernandez, 2018; Herron et al., 2003). The characteristics of lipoprotein particles, such as the diameter and concentration of the particles, can also influence the risk of CVD. Having a higher plasma concentration of particles of the large HDL subclass is strongly associated with a lower risk of CVD, while the concentration of smaller HDL particles is less protective (Freedman et al., 1998; Mora et al., 2009). Conversely, the concentration of large particles of LDL is only weakly associated with the risk of CVD, while high concentrations of smaller LDL are strongly positively related to CVD (Mora et al., 2009). The impact of smaller LDL particles on CVD is related to their greater sensitivity to oxidation compared to larger LDL particles, as oxidized LDL is a major driver of atherosclerosis development and CVD (Tribble et al., 2001). Clinical studies have examined the effects of adding dietary cholesterol via egg intake on lipoprotein particle profiles during conditions of weight maintenance and weight loss. Increases in LDL size and elevated

concentrations of large LDL particles have been observed with egg consumption, sometimes at the expense of smaller, more atherogenic LDL (Blesso, Andersen, Barona, Volek, & Fernandez, 2013; Herron, Lofgren, Sharman, Volek, & Fernandez, 2004). In addition, consumption of eggs also increased the size of HDL and the concentration of large HDL particles (Blesso et al., 2013; Greene, Waters, Clark, Contois, & Fernandez, 2006; Mutungi et al., 2010).

Many attempts have been made to reduce the cholesterol content of the egg by acting on the hen through feed, genetics or even pharmacology. All these attempts ended in failures or too limited effects. This near impossibility of reducing the cholesterol content of the egg is attributable to the fact that this molecule is associated with VLDL (Nys & Sauveur, 2004). Indeed, yolk cholesterol is transported by VLDLy and to a lesser extent by vitellogenins. Therefore, the cholesterol levels in the yolk depend on its concentration in VLDLy. The presence of free cholesterol in the surface layer is essential to maintain the structure and properties of VLDLy. Therefore, very little variation is observed for this free cholesterol. In contrast, esterified cholesterol (20% of total egg cholesterol) present in the core is more variable. Altering the structure of VLDLy would be a prerequisite for reducing yolk cholesterol, but the structure of VLDLy is quite stable due to the tight physiological controls associated with their formation (Nys & Guyot, 2011).

2.4.3. Vitamins and minerals in egg

The egg, and in particular its yolk, is a food high in vitamins A, D, E, K, and B. Consuming two eggs provides 10 to 30% of human's daily requirement for these vitamins. However, egg does not contain vitamin C (Nys & Sauveur, 2004). The vitamin concentration is influenced by genetics, rate of egg production and, just as in the case of fatty acids; it varies with the composition of the hen's diet (Leeson & Caston, 2003; Naber, 1993). The transfer efficiency of vitamins from the hen's diet to the egg is very high for vitamin A; high for riboflavin, pantothenic acid, biotin, and vitamin B_{12}; medium for vitamin D_3 and vitamin E; and low for vitamin K_1, thiamine, and folate (Naber, 1993).

Vitamin E is the common biological chain-break antioxidant found in food. Its major sources are vegetable oils and plant-derived foods. Dietary supplementation with vitamin E is commonly used in the production of n-3 PUFA enriched eggs to mitigate the oxidation of n-3 PUFA, thereby preventing the formation of undesirable off-flavours (Grobas, Méndez, Lopez Bote, De Blas, & Mateos, 2002; Ren et al., 2010).

Lutein and its stereoisomer zeaxanthin are the most common xanthophylls found in egg yolk. Xanthophylls accumulate in the macular region of the retina. They are shown to reduce the risk of developing age-related macular degeneration and cataract (Granado, Olmedilla, & Blanco, 2003; Moeller, Jacques, & Blumberg, 2000). In addition to this function, studies have shown that xanthophylls also have antioxidant properties (Granado et al., 2003; Handelman, 2001). The lutein content of the egg and therefore the colour of the yolk are influenced by the lutein concentration in the diet of the laying hen. The lutein content of the whole egg varies from about 0.3 to 1 mg/100 g. It is possible to increase the lutein in egg yolk five to eight times above regular concentrations (Leeson & Caston, 2004).

Eggs are a good source of minerals. Phosphorus, selenium, iron, and zinc in eggs show the highest proportion (~16, 29, 9 and 9%, respectively) of the recommended daily intake (Seuss-Baum, 2007). One egg contains about 1 mg of iron, the availability of which is limited to 60% in the cooked egg. The sodium content of the egg is low, especially in yolk, which can therefore be used in low-sodium diets. In addition, eggs contain a very wide range of trace elements whose contents are very varied and depend largely on the diet (Nys & Sauveur, 2004). Increasing the mineral concentration in eggs is possible and has already been applied in the production of fortified eggs, e.g. for selenium (Bennett & Cheng, 2010) or iodine (Sumaiya et al., 2016).

2.5. Conclusion

Eggs are a good source of nutrients such as proteins, lipids, vitamins and minerals, which are highly bioavailable. The composition of the egg remarkably stable and independent of breeding and feeding conditions for its major classes of constituents makes its nutritional reputation. In addition, the egg can be enriched with nutrients in high demand in human nutrition such as essential fatty acids, antioxidants and vitamins.

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Chapter 3

Conjugated linoleic and linolenic acids

3.1. Introduction

Most polyunsaturated fatty acids (PUFA) have a structure containing two or more *cis* double bonds interrupted by a methylene (-CH=CH-CH_2-CH=CH-). This methylene group is not present in certain fatty acids resulting in *cis* or *trans* conjugated double bonds (-CH=CH-CH=CH-). Conjugated fatty acid is a term that denotes positional and geometric isomers of PUFA with conjugated double bonds. The most studied conjugated fatty acids are conjugated linoleic acids (CLA) and conjugated linolenic acids (CLnA). The aim of this chapter is to summarize the current knowledge on CLA and CLnA, putting emphasis on strategies to increase their content in foods from animal origin by modifying the animal diet.

3.2. Conjugated linoleic acids

3.2.1. Occurrence and sources

CLA are positional and geometric isomers of octadecadienoic acid, also known as linoleic acid (*C18:2c9,c12*) (Figure 3.1). Ruminant milk and its derived products, as well as meat, are the main sources. Other foods (milk and meat of non-ruminant animals, eggs, etc.) contain CLA in very small amounts. Table 3.1 summarizes the concentration of CLA in milk and meat from different animal species commonly used in the human diet. Cow's milk and beef will be referred to as milk and meat respectively throughout this review, unless otherwise indicated.

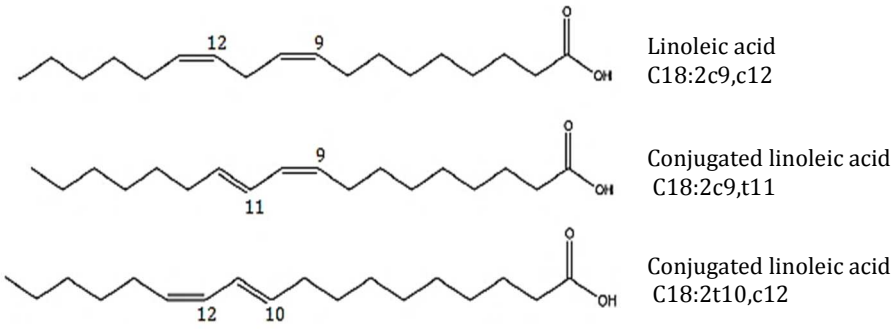

Figure 3.1. Structure of linoleic acid and two of its conjugated isomers

CLA is only 0.1 – 2% of total milk fat or tissue fat. Milk contains 3 – 18 mg CLA/g of fat, while CLA in meat represent 1 – 12 mg/g of fat (Kelly, Kolver, Bauman, Van Amburgh, & Muller, 1998; Khanal & Olson, 2004; Mir et al., 2004). Ewe's milk has a CLA content similar or even slightly higher than that of cow's milk (0.5 to 3% of fat), while goat's milk contains 2 times less (0.6 – 1.1% of fat) (Combe & Morin, 2005; Jahreis et al., 1999). Regarding ruminants' meat, lamb meat seems to be the richest in CLA and veal, the poorest (19 and 2.7 mg CLA/g of fat, respectively). The CLA content of dairy and meat products largely depends on the original CLA content in raw milk and meat. For meat products, it also depends on the nature of the meat section: lean or fatty steak, muscle or fatty tissue. CLA appear to be concentrated in intramuscular and subcutaneous fat in cattle (Mir et al., 2004).

Generally, the body fat of non-ruminants contains no more than 1% of CLA, apart from certain marsupials, such as quokka and wallaby, in which the CLA contents were found up to 2.9% and 3.7% of total fatty acids, respectively (Hartman, Shorland, & McDonald, 1955). The ruminant-like digestion in these marsupials explains the high content of conjugated dienes. Their stomach, simpler than that of ruminants, contains a rich microbial population resembling that of the rumen of sheep (Moir, Somers, Sharman, & Waring, 1954). Among non-ruminant species, turkey

meat also has a relatively high CLA content of 2 to 2.5 mg/g of fat. Chicken meat contains 0.9 mg CLA/g of fat and pork, 0.6 mg CLA/g of fat (Chin, Liu, Storkson, Ha, & Pariza, 1992; Fritsche & Steinhart, 1998; Knight, Knowles, Death, Cummings, & Muir, 2004).

Table 3.1. CLA content in milk and meat from animal species in human diet

Food	**CLA content** (in mg/g of fat)	**References**
Cow's milk	3 – 18	(Kelly et al., 1998)
Ewe's milk	5 – 30	(Combe & Morin, 2005; Jahreis et al., 1999)
Goat's milk	6 – 11	(Combe & Morin, 2005; Jahreis et al., 1999)
Sow's milk	1.9 – 2.7	(Jahreis et al., 1999)
Mare's milk	0.5 – 1.2	(Jahreis et al., 1999)
Beef	1 – 12	(Mir et al., 2004)
Lamb	5.6 – 19	(Chin et al., 1992; Knight et al., 2004)
Veal	2.7	(Chin et al., 1992)
Pork	0.6 – 1.5	(Chin et al., 1992; Fritsche & Steinhart, 1998)
Horse	0.3 – 0.9	(Dufey, 1999)
Rabbit	1.1	(Fritsche & Steinhart, 1998)
Turkey	2 – 2.5	(Chin et al., 1992; Fritsche & Steinhart, 1998)
Chicken	0.9 – 1.5	(Chin et al., 1992; Fritsche & Steinhart, 1998)
Fish	0.1 – 0.9	(Fritsche & Steinhart, 1998)

3.2.2. Biosynthesis of CLA in ruminants

In animals, two processes lead to the formation of CLA. The first is the formation of CLA as transient intermediates during the biohydrogenation of PUFA to stearic acid (*C18:0*) by the ruminal microflora in polygastrics (to a lesser extent in monogastrics by bacteria of the intestine) (Fukuda, Ninomiya, Asanuma, & Hino, 2002; Jahreis et al., 1999). The second process is the conversion in tissues of trans-vaccenic acid (*C18:1t11)* and to a lesser extent of *C18:1t7,* which are other intermediates in the biohydrogenation of PUFA, into CLA. The predominant CLA in both ruminants and non-ruminants is rumenic acid (*C18:2c9,t11*).

3.2.2.1. Biohydrogenation of polyunsaturated fatty acids in the rumen

Fodder fats are mainly composed of glycolipids and phospholipids, with linoleic acid, α-linolenic acid (*C18:3c9,c12,c15*) and γ-linolenic acid (*C18:3c6,c9,c12*) as the major fatty acids. On the other hand, lipids in oils and oilseeds used in ruminant feeds are predominantly in the form of triglycerides containing a high proportion of linoleic acid. Thus, the predominant pathways of ruminal biohydrogenation are the conversion of linoleic acid, α-and γ-linolenic acids to stearic acid (Bauman, Baumgard, Corl, & Griinari, 1999; Harfoot & Hazelwood, 1997).

The prerequisite for biohydrogenation is the hydrolysis of dietary esterified lipids (triglycerides, galactolipids, phospholipids, etc.) by microbial lipases, releasing fatty acids in the rumen. Bacterial biohydrogenation of linoleic acid is an obligatory anaerobic process involving three steps mediated by ruminal bacteria. The first reaction is the isomerization of free fatty acids present in the rumen. The 18-carbon PUFA undergo enzymatic isomerization by a linoleate *c12,t11*-isomerase, which turns the *c12* bond into *t11* (Figure 3.2). This enzyme has been localized on the cell envelope of *Butyrivibrio fibrisolvens*, a rumen bacterium. The

isomerase does not need a cofactor or a prior activation of fatty acids, but has a specific requirement for free carboxylic groups and *c9,c12* systems. The second step in the biohydrogenation pathway of linoleic acid by *B. fibrisolvens* is the hydrogenation of the *c9* bond of the conjugated diene (rumenic acid) to trans-vaccenic acid by a *c9,t11*-octadecadienoate reductase. The final step is the hydrogenation of the *t11* bond, thereby converting trans-vaccenic acid to stearic acid (Griinari & Bauman, 1999).

Rumen biohydrogenation of α and γ-linolenic acids is similar to that of linoleic acid and has trans-vaccenic acid as common intermediate (Figure 3.2). The *c12* bond is isomerized to *t11* followed by the reduction of the double bonds *c9* and *c15* (for α-linolenic acid) or *c6* and *c9* (for γ-linolenic acid) to yield trans-vaccenic acid. Finally, stearic acid is formed by the reduction of the *t*11 bond of trans-vaccenic acid (Harfoot & Hazelwood, 1997; Kepler & Tove, 1967).

Based on the reactions and end-products of biohydrogenation, two groups of bacteria (A and B) may be involved in the process of biohydrogenation of linoleic and linolenic acids. Groups A (including *B. fibrisolvens*) and B can perform the first two steps because both groups express the enzymes linoleate isomerase and CLA reductase. Only group B bacteria can saturate trans-vaccenic acid to stearic acid. The two initial steps take place quickly, while the final hydrogenation, since fewer bacteria can perform this stage, is much slower and leads to an accumulation of trans-vaccenic acid in the rumen (Kemp & Lander, 1984; Lock & Garnsworthy, 2002).

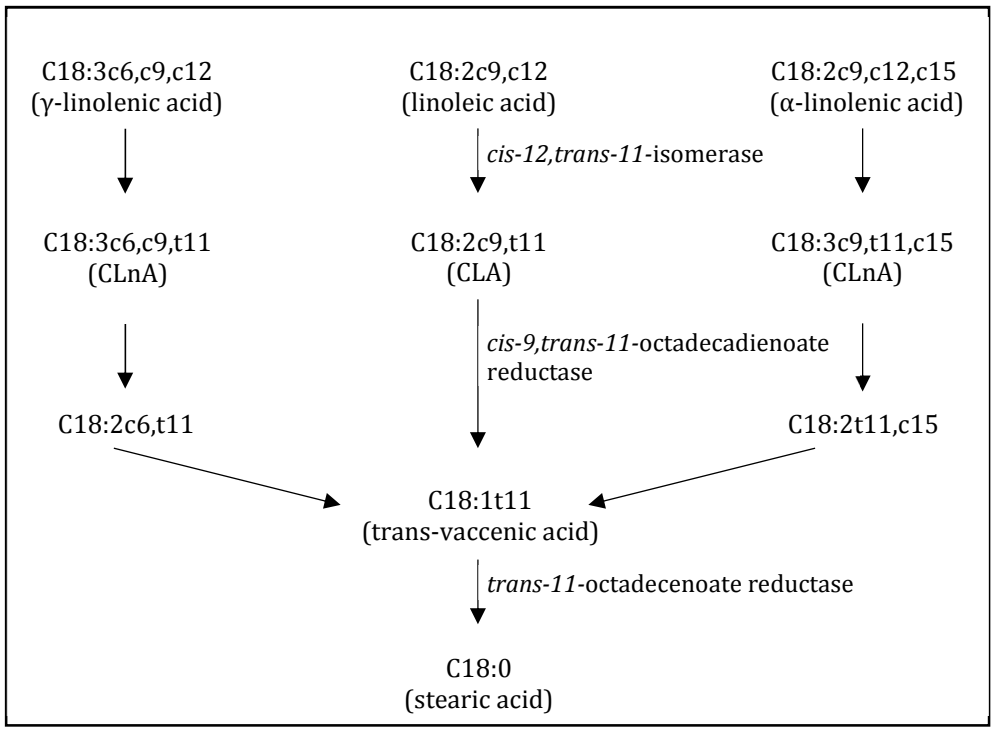

Figure 3.2. Predominant pathways of ruminal biohydrogenation of linoleic acid and α- and γ-linolenic acids (Griinari & Bauman, 1999). CLA: conjugated linoleic acid. CLnA: conjugated linolenic acid.

3.2.2.2. Endogenous synthesis of CLA in tissues

The final step of the biohydrogenation process is the slowest, leading to a transient accumulation of trans-vaccenic acid and, to a lesser extent, of other octadecenoic acids. As a result, some of these monoenes leave the rumen and escape complete biohydrogenation. They are absorbed in the intestine and then transferred to the tissues. In tissues, CLA are *de novo* synthesized by Δ9-desaturase, which introduces a *cis-9* double bond into octadecenoic acids (Griinari & Bauman, 1999). The contribution of endogenous synthesis is estimated between 64 to 91% of the overall rumenic acid content of milk, which makes it the primary source (Griinari et al., 2000; Kay, Mackle, Auldist, Thomson, & Bauman, 2004). In ruminants, adipose tissue appears to be the

major site of endogenous synthesis of CLA for growing animals. In contrast, the activity of Δ9-desaturase is highest in the mammary gland of lactating ruminants (Bauman, Baumgard, Corl, & Griinari, 1999). This endogenous synthesis of CLA explains why diets rich in α-linolenic acid also increase rumenic acid in milk. Although the biohydrogenation pathway for α-linolenic acid does not produce rumenic acid in the rumen, it leads to the formation of trans-vaccenic acid. The latter is subsequently desaturated to rumenic acid in the mammary gland, and is incorporated into the milk fat.

In theory, there are 56 possible geometric and positional isomers of CLA in *cis/cis, cis/trans, trans/cis* and *trans/trans* configurations, of which 20 isomers have been found in natural milk fat (Roach, Mossoba, Yurawecz, & Kramer, 2002; Sehat et al., 1998). Among CLA, rumenic acid is considered to be responsible for most of the health benefits and its level varies from 75 to 90% of the total CLA in milk and meat (Chin et al., 1992). Another biologically active isomer is t10,c12 CLA (*C18:2t10,c12*), which accounts for less than 5% of the total CLA in milk (Khanal & Olson, 2004).

3.2.3. Potential health benefits of CLA

Over the past four decades, numerous studies have been conducted to determine the extent of the benefits of CLA including cancer prevention, weight management, reduction and prevention of atherosclerosis and modulation of the immune system, as well as the mechanisms by which CLA intervene in these processes.

3.2.3.1. Anti-carcinogenic property

CLA have been shown *in vivo* and in *vitro* to be effective in inhibiting the initiation, promotion and progression of breast, colon, skin and prostate cancers (Field & Schley, 2004; Ip et al., 2002; Larsson, Bergkvist, & Wolk, 2005). In *in vitro* studies using

rumenic acid, the t10,c12 isomer, and a mixture of isomers, CLA showed anti-cancer activity regardless of the isomer used. However, these isomers have separate mechanisms and different targets of actions (Chujo et al., 2003; Ip et al., 2002; Masao Yamasaki et al., 2002).

Clinical trials in cancer patients are rare. Among those reported, a study showed that CLA dietary intake and serum CLA levels are inversely associated with breast cancer risk in postmenopausal women (Aro et al., 2000). In agreement, Larsson et al. (2005) observed an inverse relationship between the long-term consumption of fat-rich dairy products and the risk of developing colorectal cancer. The authors stated that this preventive effect could be due in part to ingestion of CLA via dairy products. In a randomized, double-blind, placebo-controlled trial with rectal cancer patients undergoing chemoradiotherapy, the administration of 3 g CLA/day for 6 weeks reduced inflammatory factors including tumour necrosis factor (TNF)-α and interleukin (IL)-1β, and matrix metalloproteinase (MMP)-2 and -9 enzymes, as biomarkers of angiogenesis and tumour invasion (Mohammadzadeh, Faramarzi, Mahdavi, Nasirimotlagh, & Asghari Jafarabadi, 2013).

One mechanism by which CLA exert anti-carcinogenic activity could be the activation of peroxisome proliferator-activated receptors (PPARs). Indeed, CLA have been shown to be activators of PPARα, PPARγ and PPAR δ (Bassaganya-Riera et al., 2004; Moya-Camarena, Vanden Heuvel, Blanchard, Leesnitzer, & Belury, 1999). That activation of PPARs induces apoptosis and inhibits proliferation of prostate (Kubota et al., 1998), breast (Yee et al., 2003), colon (Sarraf et al., 1999) and gastric cancer cells (Sato et al., 2000). In addition, PPAR activators can also inhibit the activation of nuclear factor (NF)-κB and activator protein 1 (AP1) (Bassaganya-Riera et al., 2004; Delerive et al., 1999). As an example, CLA have been shown to decrease cell proliferation and

inhibit the activation of NF-κB in human breast cancer cells (Rakib et al., 2013).

3.2.3.2. Anti-obesity property

The effect of CLA on overall body composition is one of the most studied fields regarding the health benefits of CLA. This is probably because obesity is a major risk factor for many diseases such as diabetes and atherosclerosis (Crumb & Vattem, 2011). Most animal and human research has supported the effectiveness of CLA supplementation in reducing weight and body fat (Blankson et al., 2000; Gaullier et al., 2007; Smedman & Vessby, 2001).

Evidence from *in vivo* and *in vitro* studies suggests that the anti-obesity effects of CLA could be attributed to increased lipolysis in adipocytes and increased β-oxidation of fatty acids in adipocytes and skeletal muscles (Pariza, Park, & Cook, 2000; Park, Storkson, Albright, Liu, & Pariza, 1999). In rats, CLA have been reported to suppress fatty acid synthesis in adipose tissue and enhance fatty acid β-oxidation in the liver (Wang et al., 2006). In addition, CLA have been shown to improve β-oxidation of fatty acids in muscle and brown adipose tissue, as well as oxygen uptake and energy expenditure in rats (Nagao et al., 2003; Rahman et al., 2001). The anti-obesity property of CLA could also be due to the activation of PPARs. CLA can increase the gene expression of fatty acid β-oxidation enzymes, including carnitine-palmitoyl transferase, acyl-CoA oxidase, and uncoupling protein in the liver, muscle, and brown adipose tissue through PPARα-dependent mechanisms (Rahman et al., 2001; Wang et al., 2006). Moreover, CLA have been shown to reduce adipose tissue by apoptosis and cause lipoatrophy in mice (Tsuboyama-Kasaoka et al., 2000). Most of these studies used a mixture of isomers of CLA. However, the anti-obesity property of CLA is attributed to the t10,c12 isomer rather than rumenic acid (Riserus, Vessby, Arnlov, & Basu, 2004; Sluijs,

Plantinga, de Roos, Mennen, & Bots, 2010; Whigham, Watras, & Schoeller, 2007).

3.2.3.3. Anti-diabetic property

Contradictory results have been published regarding the potential effects of CLA on insulin sensitivity. Whereas in obese Zucker (fa/fa) rats CLA improved glucose tolerance (Henriksen et al., 2003), they induced insulin resistance in mice (Poirier et al., 2005). There is also conflicting information on which CLA isomers may be responsible for glucose tolerance changes. The t10,c12 isomer has been reported to significantly decrease insulin sensitivity. In a double-blind placebo controlled study in obese abdominal men treated with rumenic acid, it was also observed that this isomer decreased insulin sensitivity compared to placebo (Riserus et al., 2004). This report was the first to suggest some adverse effects of rumenic acid on human health (Churruca, Fernández-Quintela, & Portillo, 2009). However, such an effect was not observed when a 50:50 (w/w) mixture of CLA isomers was given as a supplement (Riserus, Arner, Brismar, & Vessby, 2002). Another study (Tricon et al., 2004) suggested that the t10,c12 CLA increased blood glucose relative to rumenic acid. However, this effect was insufficient to modify the degree of insulin resistance or insulin sensitivity.

A study to investigate the effects of CLA in humans showed that 8-week CLA supplementation did not affect any indicator of metabolic control in overweight type 2 diabetic patients (Shadman, Taleban, Saadat, & Hedayati, 2013). Conversely, a previous study had suggested a positive effect of CLA on the reduction of insulinemia and insulin resistance in type 2 diabetics consuming a diet rich in n-3 PUFA (Schmitt et al., 2006).

The inconsistent evidence in animal and human studies makes it difficult to determine the anti-diabetic effects of CLA. Another important aspect is the contrasting functionalities of the

CLA isomers and the fact that the majority of trials have been performed with a mixture of CLA isomers. Few studies have used only one isomer of CLA. Therefore, the evidence seems insufficient and is not unanimous on the different effects of CLA.

3.2.3.4. Anti-atherosclerosis property

In some animal studies, CLA have been shown to have protective and/or therapeutic effects on sclerotic lesions associated with atherosclerosis (Arbonés-Mainar et al., 2006; Kritchevsky et al., 2004; Wilson, Nicolosi, Chrysam, & Kritchevsky, 2000). However, the results were inconsistent and the effects of CLA on atherogenesis appear to be dose-, isomer-, tissue- and species- specific. Similarly, human trials have yielded conflicting results (Sluijs et al., 2010; Sofi et al., 2010). The inconclusive results of the human and animal studies can be attributed to contrasting doses of CLA, isomers, coexistence of other dietary fatty acids, study duration and inter and/or intra-species diversities.

Most research to determine a possible mechanism of action for CLA has focused on atherosclerosis as an inflammatory condition. It has been proposed that CLA could mediate the expression of inflammatory genes, thus altering the signalling pathway that leads to the formation of reactive oxygen species (ROS). ROS not only oxidize LDL, but also negatively affect the vascular endothelium. Atherosclerosis is associated with abnormal redox-mediated signals in the vascular system. The pathways mediated by NF-κB and PPARs are the two main redox-sensitive signalling pathways involved in atherogenic and inflammatory processes. Oxidized LDL probably affects atherogenesis through activation of the NF-κB pathway. As described previously, the CLA isomers are ligands and activators of PPARs and suppress the activation of NF-κB. The mechanism by which PPARs, especially PPAR-γ activation, inhibit NF-κB-dependent cell death signals appears to involve the inhibition of the degradation of the inhibitor

of NF-κB (I-κBα), evidenced by inhibition of cytokine-induced NF-κB-dependent signalling events (Kim et al., 2007). In addition, PPARγ activation potently inhibits TNF-α- and IL-1β-induced NF-κB transcriptional activity (Remels et al., 2009). On the other hand, CLA alter the metabolism of essential fatty acids such as linoleic acid, which can be converted to arachidonic acid by elongation and desaturation. Thus, CLA, by inhibiting the synthesis of arachidonic acid, modulate the inflammatory response that is generally under the control of eicosanoids produced from arachidonic acid (Nakamura, Flintoff-Dye, & Omaye, 2008).

3.2.3.5. Immune response modulation

The effects of CLA in the modulation of immune responses can have positive implications for human health. These include reduced antigen-induced cytokine production by immune cells, decreased adverse effects of immune challenges, and modulation of inflammatory mediators such as cytokines, prostaglandins (PG), leukotrienes and immunoglobulins (Ig) (Albers et al., 2003; Turpeinen, Ylönen, von Willebrand, Basu, & Aro, 2008; Yu, Correll, & Vanden Heuvel, 2002).

Yu et al. (2002) reported that various CLA decreased the interferon (IFN)γ-induced mRNA expression of mediators of inflammation including cyclooxygenase 2 (COX2), inducible NOS (iNOS), and TNFα in mouse macrophage cells. The reduction in IFNγ-stimulated transcriptional activity leads to a decrease in the production of PGE2, TNFα and the inflammatory agent nitric oxide (NO), as well as of the pro-inflammatory cytokines IL-1β and IL-6 (Yu et al., 2002). Subsequently, rumenic acid was found to be the isomer responsible for the inhibition of lipopolysaccharide (LPS)-induced TNF-α production (Yang & Cook, 2003). CLA have also been reported to increase levels of IgA, IgG, and IgM while lowering those of IgE, thus suggesting a potential anti-allergic effect of CLA (Sugano, Tsujita, Yamasaki, Noguchi, & Yamada, 1998).

Furthermore, supplementing healthy men with rumenic acid or t10,c12 CLA significantly reduced mitogen-induced T lymphocyte activation without affecting lymphocyte subpopulations (Tricon et al., 2004).

CLA could modulate allergies and immune responses by acting at three main levels. First, CLA improve T-cell function and increase the overall efficiency of the immune system by adapting to specific antigenic responses. Second, they increase humoral function by increasing the production of IgA. Finally, CLA are involved in the function of macrophages by reducing anti-inflammatory responses with potentially involved mediators being PPARs (Crumb & Vattem, 2011; O'Shea, Bassaganya-Riera, & Mohede, 2004; Viladomiu, Hontecillas, & Bassaganya-Riera, 2016).

3.2.4. Strategies to increase dietary intake of CLA in humans

The consumption of CLA, mainly rumenic acid, in human populations varies between 15 and 1500 mg/day with marked differences between countries, genders and socio-economic groups. Milk and dairy products contribute between 66 – 80% of total CLA intake, while consumption of lamb, beef and other ruminant meat products represents 15 – 32% of average CLA intake (Shingfield & Wallace, 2014). There is no official dietary recommendations regarding CLA. Based on animal studies, it is estimated that approximately 3 g/day of CLA is required to produce beneficial effects in a 70 kg human (MacDonald, 2000; Sailas, Prakasan, Sreedharan, Wright, & Spener, 2015). Obtaining adequate amounts from ruminant-derived products could be difficult because of their low concentrations in CLA. In addition, increased consumption of milk and meat to reach physiologically active levels of CLA would also result in a high intake of saturated fatty acids (SFA), which are considered to be detrimental to cardiovascular health. Thus, an effective solution for improving the

CLA status of humans is by using oral supplements or by enriching foods derived from non-ruminants with CLA.

3.2.4.1. CLA dietary supplements

CLA marketed as dietary supplements are produced chemically from vegetable oils. The basic process for preparing CLA from vegetable oil is the isomerization of the double bonds of linoleic acid in the presence of an alkaline solution at high temperature. The starting oil should contain a high percentage of linoleic acid such as safflower, sunflower or soybean oil (Kim, Lee, Park, Kim, & Ha, 2000; Salamon, Vargáné-Visi, András, Csapóné Kiss, & Csapó, 2015).

CLA dietary supplements provide larger proportions of CLA than milk and meat. They are therefore taken to compensate for the lack of CLA provided by the diet and especially to obtain high doses. The actual amount of CLA in these products varies between brands and ranges from 300 to 1000 mg per serving. Dietary CLA supplements are mainly composed of rumenic acid and t10,c12 isomer, which can together account for more than 80% of the total CLA (Sailas et al., 2015). Analysis of commercial CLA products revealed great differences in total CLA content, isomer distribution and fatty acid composition depending on the original oil used to prepare the CLA product and on the reaction conditions during the production (Yu, Adams, & Watkins, 2003; Yurawecz et al., 1999).

3.2.4.2. CLA-enriched foods from non-ruminants

Though substantial amounts of CLA are not present in foods from non-ruminants, several studies have shown that supplementing the diet with synthetic mixture of CLA induces an increase in their content in chicken eggs, in the muscle and adipose tissue of pigs, broilers and fishes. However, despite the potential for enhancing nutritional properties that can be achieved, animal

products enriched in CLA are not yet widely marketed. Moreover, increasing the CLA content in meat may alter its textural and sensory properties to a certain extent depending on the animal species.

i. Chicken eggs

Feeding laying hens with CLA-enriched diets allows a dose-dependent deposition of CLA isomers in the lipids of egg yolk. Chamruspollert and Sell (1999) reported that the CLA concentrations in yolk lipids of hens fed 0.5, 2.5 or 5.0% CLA mixture by weight of feed were 0.82, 5.82, and 11.20% of total fatty acids, respectively. CLA supplementation not only increases the CLA content in egg yolk, but also leads to an increase in SFA and a decrease in monounsaturated fatty acids (MUFA) by down-regulating the activity of stearoyl-CoA desaturase (SCD) or Δ9-desaturase (Chamruspollert & Sell, 1999; Du, Ahn, & Sell, 1999; Shang, Wang, Li, Yin, & Li, 2004). In addition to the alteration of the fatty acid profile of the yolk, the CLA-enriched diet would increase the permeability of the yolk membrane. Ahn and coinvestigators (1999) noted an increase of the firmness of hard-cooked yolk following a refrigerated storage, as well as a rubbery and elastic texture. They speculated that the quality changes of CLA-enriched eggs could be related to the increase of water content of the yolk, the changes of yolk pH and the movement of ions between yolk and albumen across yolk membrane during storage (Ahn, Sell, Jo, Chamruspollert, & Jeffrey, 1999). For some authors, dietary CLA would be accompanied by a decrease in the size of yolks and eggs (Chamruspollert & Sell, 1999; Shang et al., 2004), or by a significant decrease in the egg production (Shang et al., 2004; Szymczyk & Pisulewski, 2003).

ii. Chicken meat

Szymczyk et al. (2001) investigated the effect of a dietary mixture of CLA on growth performance, carcass composition, fatty acid composition of adipose and muscle tissues in broilers. They reported a linear increase in CLA concentrations in tissue lipids when feeding chickens with incremental levels of CLA. At the same time, broilers fed on CLA supplemented diets exhibited impaired growth performance and a significant decrease in abdominal fat deposition. The proportion of breast muscles was not affected while that of the legs increased. Among other effects, dietary supplementation with CLA negatively affected the fatty acid composition of tissues by increasing their SFA content at the expense of MUFA and PUFA. Poultry meat enriched with CLA exhibited some alterations in its quality, such as an increase in the hardness and darkness of the meat, and a decrease in juiciness (Szymczyk, Pisulewski, Szczurek, & Hanczakowski, 2001). Du and Ahn (2002) reported that the increase in hardness and decrease in juiciness may be due to changes in the fatty acid composition of muscle lipids. The higher proportion of SFA in muscle tissue enriched with CLA and the consequent decrease in MUFA and PUFA increase the melting point of fats, which would make the meat drier and harder. Another explanation is that the protein content increased in CLA-enriched poultry carcasses, which increased the hardness of the meat (Du & Ahn, 2002).

iii. Pork

The ingestion of synthetic CLA in pigs is associated with a decrease in fat deposition and an increase in lean tissue. Some works have also observed a slight improvement in the growth rate of growing pigs (Ostrowska, Muralitharan, Cross, Bauman, & Dunshea, 1999; Ostrowska et al., 2003). Dietary CLA change the fatty acid composition of pork. The total content of unsaturated fatty acids decreases, while CLA and SFA increase. Joo et al. (2002)

reported that a CLA-enriched diet in pigs improved the colour stability of meat, decreased lipid oxidation and increased the water-holding capacity of pork loin as well as intramuscular fat content.

iv. Fish

In fish, diet supplementation with synthetic CLA has the effect of increasing the content of CLA and SFA in the tissues, and decreasing that of MUFA and PUFA (Twibell, Watkins, & Brown, 2001). Tan et al. (2014) indicated that weight gain and growth rate decreased with high levels of dietary CLA in fish. They also observed that whole body fat content decreased with dietary CLA, while the hepatic fat content increased.

3.3. Conjugated linolenic acids

CLnA are positional and geometric isomers of α-linolenic acid (Figure 3.3). Some CLnA are dienes characterized by two conjugated double bonds like CLA, while others possess three conjugated double bonds. The former are generated through the isomerization of α-linolenic acid by bacteria from the gut of ruminants and are sometimes called enteric CLnA (Białek, Czauderna, & Białek, 2017). The latter are typical of some seed oils (Koba, Belury, & Sugano, 2007). CLnA isomers have been attributed with several health benefits including anti-carcinogenicity, anti-obesity and anti-oxidant activity. However, compared to CLA, studies dealing with physiological effects of CLnA are limited.

3.3.1. Occurrence and sources

CLnA of enteric origin exist naturally in milk fat and ruminant meat. However, the level of CLnA in these foods is around 0.03% (Destaillats, Trottier, Galvez, & Angers, 2005). This makes it

impossible to use ruminant fat as an effective dietary source of CLnA. By contrast, CLnA with three conjugated double bonds occur mainly in the seed oils of certain plants, and they are usually most prevalent among fatty acids. Five CLnA isomers, namely α-eleostearic (*C18:3c9,t11,t13*), punicic (*C18:3c9,t11,c13*), α-calendic (*C18:3t8,t10,c12*), jacaric (*C18:3c8,t10,c12*), and catalpic (*C18:3t9,t11,c13*) acids, are found as the major component of seed oils of several plants. Among the CLnA-containing seeds listed in Table 3.2, only the seeds of *Neocarya macrophylla*, *Punica granatum*, *Ricinodendron heudelotii* and *Trichosanthes kirilowii* are edible for humans.

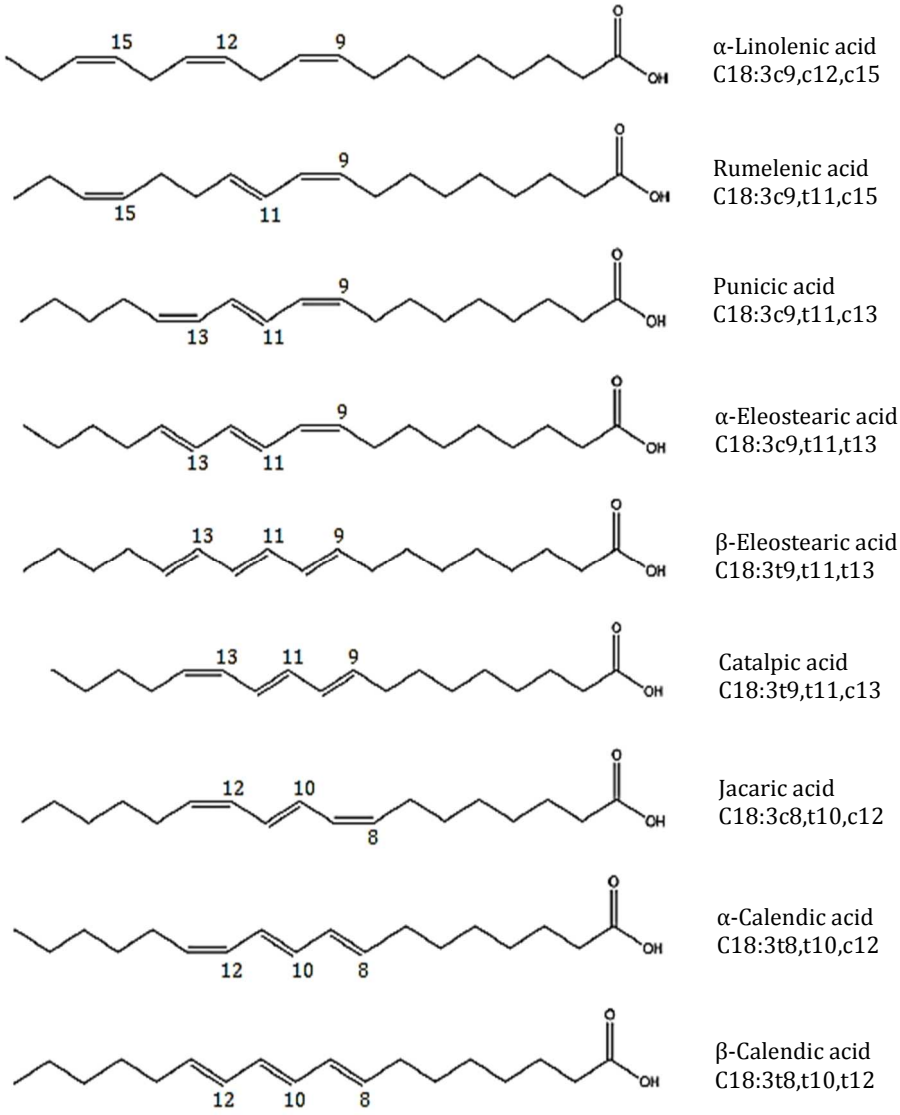

Figure 3.3. Structure of α-linolenic acid and some of its conjugated isomers

Table 3.2. CLnA content in some plant seed oils

Plants	**Major CLnA isomer**	**CLnA content** % (w/w) fatty acids	**References**
Aleutrites fordii	α-Eleostearic acid (C18:3c9,t11,t13)	60 - 70	(Koba et al., 2007; Takagi & Itabashi, 1981)
Calendula officinalis	α-Calendic acid (C18:3t8,t10,c12)	50 - 62	(Takagi & Itabashi, 1981)
Catalpa ovata	Catalpic acid (C18:3t9,t11,c13)	30 - 42	(Koba et al., 2007; Takagi & Itabashi, 1981)
Jacaranda mimosifolia	Jacaric acid (C18:3c8,t10,c12)	30 - 40	(Koba et al., 2007; M. Yamasaki et al., 2013)
Momordica basalmina	Punicic acid (C18:3c9,t11,c13)	50	(Gaydou, Miralles, & Rasoazanakolona, 1987)
Momordica charantia	α-Eleostearic acid (C18:3c9,t11,t13)	40 - 60	(Koba et al., 2007; Takagi & Itabashi, 1981)
Momordica cochinchinensis	α-Eleostearic acid (C18:3c9,t11,t13)	59 - 65	(Hopkins, Chisholm, & Orgodnik, 1969)
Neocarya macrophylla	α-Eleostearic acid (C18:3c9,t11,t13)	27 - 33	(Miralles et al., 1994)
Punica granatum	Punicic acid (C18:3c9,t11,c13)	70 - 85	(Aruna, Venkataramanamma, Singh, & Singh, 2016)
Ricinodendron heudelotii	α-Eleostearic acid (C18:3c9,t11,t13)	46 - 51	(Nzali, Tchiegang, Sandjon, & Meurens, 2016; Tchiégang, Kapseu, Ndjouenkeu, & Ngassoum, 1997)
Tricosanthes kirilowii	Punicic acid (C18:3c9,t11,c13)	40	(Koba et al., 2007)

3.3.2. Biosynthetic origin of CLnA

3.3.2.1. Formation of CLnA in plant seeds

Conjugated double bonds found in fatty acids of vegetable oils have been shown to result from the modification of an existing *cis* double bond. However, the mechanisms involved in these reactions have yet to be determined, although a number of biosynthetic scenarios have been proposed.

Cahoon et al. (1999) have demonstrated that the conjugated *trans*-Δ11- and *trans*-Δ13 double bonds of α-eleostearic acid in the seed of *Momordica charantia* are synthesized by divergent forms of the Δ12-oleic-acid desaturase (fatty acid desaturase 2 enzyme, FAD2), called "conjugases". These enzymes catalyze the conversion of an existing *cis*-Δ12-double bond into conjugated *trans*-Δ11- and *trans*-Δ13-double bonds. Their activity contrasts with that of the typical FAD2 of plants, which introduces a *cis*-Δ12-double bond into oleic acid (Cahoon et al., 1999; Liu, Hammond, & Nikolau, 1997). The reaction uses the fatty acid bound to phosphatidylcholine as substrate, as has been shown for other FAD2-type enzymes (Liu et al., 1997; Shanklin & Cahoon, 1998). Different plants appear to have different conjugase enzymes, leading to the conversion of linoleic acid into different CLnA isomers. For example, a class of FAD2-related enzymes that modifies a Δ9-double bond to produce the conjugated *trans*-Δ8- and *trans*-Δ10-double bonds found in calendic acid has been identified in *Calendula officinalis* (Cahoon, Ripp, Hall, & Kinney, 2001).

3.3.2.2. Biohydrogenation of α-linolenic acid in ruminants

As previously described (Section 3.2.2.1), the main pathway for biohydrogenation of α-linolenic acid by the anaerobic microbial population in the rumen starts with the isomerization of α-linolenic acid via the activity of a *c12,t11*-isomerase, leading to *C18:3c9,t11,c15* (rumelenic acid). Then, the *cis*-Δ9-double bond is

hydrogenated to produce a non-conjugated dienoic fatty acid (*C18:2t11,c15*), followed by the reduction of *t11* and *c15* to produce stearic acid (Figure 3.2).

Numerous studies have shown the complexity of α-linolenic acid biohydrogenation in the rumen. The results showed that rumen microbes are able to biohydrogenate α-linolenic acid via alternative pathways, resulting in many CLA and CLnA isomers that differ in geometry and location of double bonds, including their conjugated configuration (Destaillats et al., 2005; Lee & Jenkins, 2011; Shingfield, Bernard, Leroux, & Chilliard, 2010).

3.3.3. Bioconversion of CLnA to CLA

Several studies have reported that α-eleostearic acid and punicic acid can be metabolized into rumenic acid in rats, mice and humans (Tsuzuki et al., 2006; Tsuzuki et al., 2004; Yuan, Sinclair, Zhou, & Li, 2009; Yuan, Sinclair, Xu, & Li, 2009). Suzuki and colleagues have also shown that administration of catalpa seed oil, containing more than 40% catalpic acid, to rats, induced the accumulation of t9,t11-CLA in the liver and colon mucosa (Suzuki et al., 2006). However, studies conducted by Shinohara and coworkers on mice fed jacaric acid showed no accumulation of CLA in the tissues (Shinohara et al., 2012). The difference between jacaric acid and the previously mentioned CLnA isomers lies in the 8,10,12 position of the three double bonds and, in turn, in the absence of the Δ13 double bond. The mechanism by which the 9,11,13-CLnA isomers are converted to CLA remains unclear. Nevertheless, some authors suggested a mechanism, which implies that the Δ13 double bond of these CLnA is reduced by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme. Tsuzuki et al. (2006) speculated that this conversion is carried out by either a novel enzyme recognizing fatty acids with three conjugated double bonds, or an enzyme active in the reductive pathway of leukotriene B4.

Schneider et al. (2013) have shown in Caco-2 cells that the conversion rate differs depending on the CLnA isomer. This conversion appeared to be lower for CLnA having a Δ13 double bond in *cis* configuration compared to those with a Δ13 double bond in *trans* configuration. Moreover, comparing isomers of the same Δ13 double bond configuration, the conversion rate was lower when the Δ9 double bond was in its *cis* configuration. The first observation has been also made in mice and rats (Tsuzuki et al., 2006; Yuan et al., 2009).

3.3.4. Potential health benefits of CLnA

3.3.4.1. Anti-carcinogenic property

Several studies have confirmed the cytotoxic effects of CLnA on animals and various tumor cell lines. The conjugated triene types exhibited a more obvious cytotoxic effect than the conjugated diene types (Igarashi & Miyazawa, 2000). Moreover, it appears that the position and geometry of the three conjugated double bonds affect their cytotoxic activity. The cytotoxic effect of four positional and geometrical CLnA isomers were compared in human leukemia cells. It was found that the cytotoxicity of 9,11,13-CLnA isomers, α-eleostearic, punicic and catalpic acids, is much stronger than that of α-calendic acid, an 8,10,12-CLnA isomer (Suzuki et al., 2001).

Yasui et al. (2006) showed in the human colon cancer cells Caco-2 that β-eleostearic and β-calendic acids with *all-trans* conjugated double bonds exerted stronger growth inhibition and more DNA fragmentation, an indicator of apoptosis induction, than α-eleostearic and α-calendic acids, which have one double bond in *cis* configuration. In addition, the cytotoxic effects of β-eleostearic and β-calendic acids were not completely counteracted by the antioxidant α-tocopherol, whereas the cytotoxic effects of α-eleostearic and α-calendic acids were lost in the presence of α-tocopherol. The authors indicated that β-eleostearic and β-calendic

acids may have signaling pathways different from those of α-eleostearic and α-calendic acids.

Gasmi and Sanderson (2013) investigated the ability of CLnA isomers to induce apoptosis in various types of human prostate cancer cells. CLnA selectively induced apoptosis in hormone-dependent (LNCaP) and -independent (PC-3) human prostate cancer cells, without affecting the viability of normal human prostate epithelial cells. The *cis*/*trans*/*cis* configuration of jacaric and punicic acids was shown to play a key role in their increased potency and effectiveness, as compared to α- and β-calendic acids, which were also tested. Jacaric acid induced concentration- and time-dependent LNCaP cell death through activation of intrinsic and extrinsic apoptotic pathways. This resulted in cleavage of PARP-1, modulation of the pro- and anti-apoptotic Bcl-2 family of proteins and increased cleavage of caspase-3, -8 and -9. It also led to the activation of a signaling cascade inducing cell death involving the death receptor 5 (Gasmi & Sanderson, 2013).

Lipid peroxidation appears to play an important part in the cytotoxicity of CLnA on cancer cells. As mentioned above, Yasui and coworkers (2006) highlighted that the cytotoxic effect of CLnA was totally or partly abolished by α-tocopherol. In 2010, Grossman et al. reported that the cytotoxicity of CLnA involves lipid peroxidation, which causes activation of protein kinase C (PKC) responsible for inducing apoptosis and inhibiting proliferation (Grossmann, Mizuno, Schuster, & Cleary, 2010). Partly in line with these publications, a very recent study conducted by Beatty and colleagues (Beatty et al., 2021) showed that the CLnA isomer α-eleostearic acid triggers cell death in triple negative breast cancer cells through ferroptosis. Ferroptosis is a form of iron-catalyzed regulated cell death that is morphologically, biochemically and genetically distinct from other regulated cell deaths, such as apoptosis and necroptosis (Dixon et al., 2012).

3.3.4.2. Anti-obesity property

Koba et al. (2007) examined the effects of some individual CLnA isomers in rats using bitter gourd oil, pomegranate seed oil (PSO), catalpa oil and calendula oil. This study showed that feeding PSO containing punicic acid but not the other CLnA-rich oils resulted in a significant decrease in the weight of perirenal adipose tissue compared to the feeding with control oil (α-linolenic acid-rich linseed oil). The authors reported that the anti-obesity effect could be at least partly due to an increase of fatty acid β-oxidation in the liver and brown adipose tissue. This effect is attributed to punicic acid itself rather than its CLA metabolite, because both α-eleostearic acid in bitter gourd oil and punicic acid in PSO can be converted to rumenic acid, but only the latter showed a reduction in the adipose tissue weight. In a 12-week mice study, consumption of PSO prevented diet-induced obesity by decreasing body fat in animals (Vroegrijk et al., 2011).

3.3.4.3. Anti-diabetic property

Findings of various *in vivo* and *in vitro* studies regarding the efficacy of the anti-diabetic activity of CLnA, in particular punicic acid, are controversial. Some investigations suggest that PSO may improve type 2 diabetes by ameliorating high fat diet-induced obesity and insulin resistance in mice (Vroegrijk et al., 2011). In an *in vitro* study, punicic acid improved fasting plasma glucose by activating PPARα and γ (Hontecillas, O'Shea, Einerhand, Diguardo, & Bassaganya-Riera, 2009). In a randomized double-blind clinical trial study, the consumption of 2 g PSO per day for 8 weeks had no effect on fasting blood glucose and insulin resistance in diabetic patients (Faghihimani, Mirmiran, Sohrab, Iraj, & Faghihimani, 2016).

3.3.4.4. Antioxidant activity

Saha and coinvestigators conducted a series of *in vitro* and *in vivo* studies to investigate the antioxidant activity of CLnA isomers against chemically induced oxidative stress. While evaluating the antioxidant activity of vegetable oils containing α-eleostearic acid and punicic acid *in vitro*, they observed that the two CLnA isomers exerted a better antioxidant activity at lower concentrations (125 mg/ml) because of the better oxidative stability. Alpha-eleostearic acid showed more prominent antioxidant activity than punicic acid due to the presence in the molecule of a higher number of *trans* double bonds (Saha, Patra, & Ghosh, 2012). In rats suffering from oxidative stress induced by sodium arsenite, α-eleostearic and punicic acids enhanced the activities of superoxide dismutase, catalase and glutathione peroxidase, and decreased the activity of nitric oxide synthase at normal levels in the liver, plasma and brain (Saha & Ghosh, 2010; 2011). Both isomers improved renal oxidative stress and when administered together the isomers showed synergistic activity (Saha & Ghosh, 2013). However, it has been shown that depending on the doses used, punicic acid can act as pro-oxidant (at 1.2% punicic acid by weight of diet) or antioxidant (at 0.6% punicic acid by weight of diet) (Mukherjee, Bhattacharyya, Ghosh, & Bhattacharyya, 2002).

3.3.5. Strategies to increase dietary intake of CLnA in humans

Historically, conjugated vegetable oils such as tung (*Aleutrites fordii*) oil are used as drying oils added to paints, varnishes and inks, and as resins or binders on wood and wood products. Due to their conjugated double bonds, which make them highly reactive towards polymerisation, conjugated oils exhibit faster drying rates, better water resistance and improved toughness compared to unconjugated oils (Philippaerts, Goossens,

Jacobs, & Sels, 2011; Quirino, 2014; Sleeter, 1996). Recently, much attention has been paid to CLnA for food applications, due to promising results referring to their relevant physiological activities. Based on the results obtained from animal studies, a dietary intake of 2 – 3 g of CLnA /day has been estimated as the effective and safe dose for humans (Shinohara et al., 2012). The easiest way to provide sufficient amounts of CLnA for physiological benefits is by consuming CLnA-rich vegetable oil. Unfortunately, most of these oils are not stable because of their high oxidability. Furthermore, efforts to increase the content of enteric CLnA in milk and meat resulted in only minor improvement (Białek et al., 2017).

Interestingly, a recent study showed that CLnA can be incorporated into egg yolk by feeding hens with a seed oil rich in CLnA. From the research reported, a diet containing 1.5% punicic acid resulted in about 4% rumenic and 1% punicic acid in egg lipids (Kostogrys et al., 2017). This suggests that manipulating the diet by feeding the hens with an oil rich in CLnA is a way to enrich the eggs with CLA and CLnA and increase the intake of these fatty acids in humans.

3.4. Conclusion

CLA are mainly present in products from ruminants due to the action of rumen microorganisms in the biohydrogenation of PUFA. In monogastric animals, the dietary supplementation with synthetic CLA is effective in increasing the CLA content of derived foods. The discovery that animal cells are able to convert CLnA to CLA offers an alternative way to enrich food from animal origin with CLA and CLnA. Such an enrichment strategy deserves to be studied in detail in order to provide consumers with natural food products rich in health-promoting fatty acids.

3.5. References

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Chapter 4

Objectives of the thesis

The imbalance between the high intake of saturated fatty acids and omega-6 polyunsaturated fatty acids (n-6 PUFA) and low intake of omega-3 (n-3) PUFA in the modern human diet parallels a significant increase in the prevalence of overweight and obesity as well as many diseases, including cardiovascular diseases, type 2 diabetes, inflammatory diseases, and cancers. In line with this observation, several studies indicate that n-3 PUFA could be useful in the prevention of these pathologies (Bowen, Harris, & Kris-Etherton, 2016; Calder, 2017; Harris et al., 2021). In addition, the long-chain n-3 PUFA called docosahexaenoic acid (DHA) is important to mitigate inflammatory reactions and to ensure brain structure and function, more specifically during development and aging (Avallone, Vitale, & Bertolotti, 2019; Brenna & Carlson, 2014). Producing functional foods enriched with n-3 fatty acids has been adopted in recent years as a potential solution to improve n-3 PUFA in the human diet, and there are now many products enriched with n-3 PUFA on the market.

Chicken egg is one of the few foods of animal origin that is consumed by many people around the world. Its popularity is justified not only because it is easy to produce and has so many uses in cooking, but also because it contains almost all the nutrients essential for sustaining life; hence its role of total life support for the embryonic chick. In addition, the fatty acid composition of the egg can be easily modified depending on the hen's diet (Nys & Sauveur, 2004). This means that eggs can be enriched with n-3 PUFA, provided that there is an adequate supply of these fatty acids in the hen's diet.

The hen is able to incorporate as is the ingested fatty acids in its tissues. For example, by feeding animals with flaxseeds, the level of α-linolenic acid (ALA) is increased approximately twenty to forty-fold in eggs, ten-fold in chicken meat, two-fold in beef and six-fold in pork (Bourre, 2005). Hens also have an ability to convert ALA to DHA, with an estimated efficiency of 15%, leading to an accumulation of significant amounts of DHA, which can be

multiplied by five in eggs and go from 20 to 100 mg / egg (Ren, Wu, & Renema, 2010). Poultry products are therefore considered an excellent way to positively alter the nutritional status in n-3 PUFA in humans (Van Elswyk, Hatch, Stella, Mayo, & Kubena, 1998). The overall objective of this work is to obtain higher nutritional and health benefits from eggs by improving their lipid profile not only with n-3 PUFA, but also with potent bioactive fatty acids, namely conjugated linoleic acids (CLA) and conjugated linolenic acids (CLnA), through hens' feeding. The literature reports several studies that have shown that feeding hens with CLA induced the accumulation of these fatty acids in eggs. Diet containing 5% CLA could result in about 15% CLA in egg lipids (Du, Ahn, & Sell, 1999). Besides, the evidence that animal cells are capable of converting some CLnA to CLA suggests that CLnA can also be used to enrich foods of animal origin with CLA.

Our research has focused on the hypothesis that foods rich in CLnA are an indirect source of CLA for animals. More specifically, we hypothesized that the laying hen was able to perform an efficient conversion of CLnA into CLA, as is the case for the conversion of ALA into DHA, and could in turn accumulate CLA together with DHA in egg yolk. To achieve our goals, different models of diets enriched with CLnA and ALA have been developed. Samples were taken from the fed hens as well as from the eggs, and fatty acid composition analyses were performed with attention to rumenic acid (RmA) as a CLA isomer with beneficial effects, and DHA. To our knowledge, there is no information in the literature concerning the proposed research.

The first challenge addressed by this thesis was the study of the accumulation of RmA in the eggs of laying hens fed with natural sources of CLnA. Chapter 5 describes two experiments. The first one aimed at comparing the accumulation of RmA in eggs following the feeding of laying hens with two edible sources of CLnA, namely *Punica granatum* (pomegranate) seed oil rich in punicic acid (PunA) and *Ricinodendron heudelotii* kernel oil rich in α-eleostearic

acid (α-ESA). The other experiment assessed whether increasing amounts of CLnA in the feed of hens would affect the conversion of the ALA contained in the feed into DHA, and thus their respective amounts in the egg yolk.

In a second challenge, the egg enrichment strategy was evaluated by long-term feeding of hens. This experiment, described in Chapter 6, examined the persistence of n-3 PUFA, CLnA and CLA fatty acids in eggs and the stability of their content as a function of the duration of feeding with flaxseeds and pomegranate seed oil. In addition, we determined the effect of dietary lipids on the fatty acid composition of hens' tissues.

As noted in the literature review, many health benefits are attributed to the CLnA and the CLA. However, their dietary intake is not sufficiently guaranteed on a daily basis to reach physiologically active amounts. The eggs produced previously meet a significant portion of the recommended n-3 PUFA requirements and provide an appreciable amount of CLnA and CLA. Thus, the third challenge of this thesis was to study the impact of the daily consumption of these eggs on human health, in particular the potential effects of the combination of these fatty acids on the development of certain pathologies related to lipid and glucose metabolism. This clinical trial, discussed in Chapter 7, assessed the extent to which eggs enriched with n-3 PUFA, CLnA and CLA can be integrated into a common diet and contribute to improve consumers' health.

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Chapter 5

Effect of the dietary combination of flaxseed and *Ricinodendron heudelotii* or *Punica granatum* seed oil on the fatty acid profile of eggs

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Foreword

As presented in Chapter 3, feeding hens with an oil rich in CLnA can enrich their eggs with CLnA and CLA. However, little is known about the metabolism of CLnA in hens. Therefore, in this chapter, we studied the process of egg enrichment upon supplementing hens' diet with two different sources of CLnA. We evaluated the rate of incorporation of CLnA in the feed necessary to achieve a substantial deposition of CLnA and CLA in the eggs, without harming the hens. In addition, we have combined flaxseeds and CLnA oils in the feed of the hens to produce eggs of high nutritional quality. These results have been published in *Food Chemistry*. As additional data, we showed the changes in the fatty acid composition of the eggs we produced compared to that of standard unenriched eggs found in the markets.

Abstract

The health promoting omega-3, -7, and -5 fatty acids, α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA), and α-eleostearic acid (α-ESA)/punicic acid (PunA), are not currently combined in frequently consumed food items. We have evaluated the impact of supplementing laying hens' feeds with flaxseeds combined with oil derived from seeds of either *Ricinodendron heudelotii*, an α-ESA source, or *Punica granatum*, a PunA source, on the egg fatty acid profile. The supplemented diets increased the egg content in ALA, DHA, RmA, as well as α-ESA or PunA. The combination of dietary lipids did not affect the conversion rate of ALA into DHA. Hens fed on *R. heudelotii* or *P. granatum* seed oil both accumulated RmA in egg yolk, indicating an efficient conversion from the α-ESA or PunA precursors through a Δ-13 reductase activity. The accumulation of PunA in eggs was largely higher than that of α-ESA.

Keywords: Rumenic acid; α-eleostearic acid; punicic acid; docosahexaenoic acid; α-linolenic acid; enriched eggs.

5.1. Introduction

Numerous health benefits on cardiovascular diseases, central nervous system and mental health diseases, inflammation and immune functions, as well as certain types of cancer, have been reported for omega-3 polyunsaturated fatty acids (n−3 PUFA). These health benefits are mainly attributed to eicosapentaenoic acid (EPA, *C20:5c5,c8,c11,c14,c17*) and docosahexaenoic acid (DHA, *C22:6c4,c7,c10,c13,c16,c19*), rather than α-linolenic acid (ALA, *C18:3c9,c12,c15*), which is essential to serve as a substrate for the synthesis of EPA and DHA (Guo, Sinclair, Kaur, & Li, 2018). In humans, the conversion of ALA to its long-chain derivatives EPA and DHA has been estimated at less than 6% and 1% respectively,

and depends on the concentration of n-6 fatty acids in the diet (Brenna, 2002). Moreover, it is substantially lower in men (2.5-fold and 200-fold, respectively) than in women (Burdge, 2006). ALA cannot be synthetized by humans, so its dietary intake, as well as that of DHA and to a lesser extent of EPA, are essential to ensure good health. The Belgian Superior Health Council recommends an intake of ALA of 2.8 and 2.2 g/day for adult men and women respectively, based on daily energy requirements of 2500 kcal for men and 2000 kcal for women. The recommended minimal daily allowances are 250 mg/day for DHA and 500 mg/day for EPA + DHA, regardless of gender (SHC, 2016). A recent food consumption survey carried out in Belgium revealed that the population aged 3 to 64 consumes, on average per day, 1.5 g of ALA and 190 mg of EPA + DHA (De Ridder K et al., 2016). In this respect, the consumption of n-3 PUFA-enriched products, and more specifically DHA-enriched products, should be encouraged to reach the recommended intakes.

As an important component of the human diet, eggs are one of the few original and natural foods consumed throughout the world. The fatty acid profile of hen's eggs is largely impacted by the diet of the hen, which uses a significant part of the ingested fatty acids for deposition in the egg yolk. Among others, the intestinal physiology of birds has been shown to allow relatively good preservation of the dietary PUFA, leading to an increase of these fatty acids in tissues once they are supplied in the diet (Chanmugam et al., 1992). This feature has been exploited over the past 20 years to produce eggs enriched in n-3 PUFA. Feeding hens on a diet containing 20% by weight (wt.) of flaxseed leads to an ALA enrichment of up to 500 mg per egg and also increases the DHA content up to 80 mg per egg, which shows that laying hens are efficient at converting ALA to DHA (Ferrier et al., 1995). Eggs from hens fed fish oil and marine algae are mostly enriched with DHA, while EPA contents increase to a lesser extent. A DHA enrichment greater than 200 mg per egg can be obtained by supplementing a diet with 4.8% wt. of marine algae (Herber & Van Elswyk, 1996).

Next to n-3 PUFA, conjugated linoleic acids (CLA) have received considerable attention since the 1980s, due to the numerous evidence of their beneficial health properties. These include inhibition of carcinogenesis, reduction of body fat accretion, reduction in the development of atherosclerosis, anti-diabetic effects, modulation of the immune system and of the synthesis of eicosanoids (Sailas, Prakasan, Sreedharan, Wright, & Spener, 2015). Recently, the interest for conjugated linolenic acids (CLnA), especially those with three conjugated double bonds, has been growing because of the promising results from studies investigating their anti-cancerous (Dhar Dubey, Sharma, & Kumar, 2019) and anti-diabetic (Nekooeian, Eftekhari, Adibi, & Rajaeifard, 2014) properties.

Human intake of CLA predominantly comes from dairy products and ruminant meat, whose CLA concentrations typically range from 1 to 12 mg/g fat, i.e. less than 2% of the total fat content. The main CLA considered to be responsible for most of the health benefits is rumenic acid (RmA, *C18:2c9,t11*). It accounts for 75 to 90% of total CLA in ruminant derived products (Chin, Liu, Storkson, Ha, & Pariza, 1992). In contrast, CLnA are the predominant fatty acids in some plant seed oils. Among the edible seeds containing CLnA are *Punica granatum* (pomegranate) seeds and *Ricinodendron heudelotii* kernels. Pomegranate seed oil can contain up to 80% of punicic acid (PunA, *C18:3c9,t11,c13*), whereas α-eleostearic acid (α-ESA, *C18:3c9,t11,t13*) makes up to 52% of fatty acids in *R. heudelotii* kernel oil.

No official dietary recommendation has been made regarding CLA. Based on animal studies, it is estimated that an intake of approximately 3 g/day of CLA is required to produce beneficial effects in a 70 kg human (Sailas et al., 2015). Unfortunately, most of these studies have been conducted with synthetic CLA mixtures containing mainly RmA and the *C18:2t10,cis12* isomer, and the effective doses to obtain the benefits of each individual isomer are still unknown. On the other hand,

there is no suggested recommendation for CLnA. Considering that some CLnA, such as α-ESA and PunA, can be converted to RmA through a Δ13 reductase activity in animal and human tissues (liver, adipose tissue, heart, kidney, spleen, intestine, etc.) (Schneider, Mignolet, Schneider, & Larondelle, 2013; Yuan, Sinclair, Xu, & Li, 2009a), it is conceivable that some of the health benefits of CLnA are related to their conversion into RmA.

The current intake of RmA in humans varies. The dietary intake of total CLA, including RmA, has been evaluated below 500 mg/day (Ritzenthaler et al., 2001). It varies widely according to the diet, and especially depends on the intake of ruminant-derived products. In addition, the current consumers' preference for low-fat dairy products, lean cuts of beef, meat from monogastric animals such as pork and poultry, and plant-based protein alternatives, is likely to further limit the dietary intake of CLA, especially RmA. An alternative to achieve physiologically active CLA concentrations would be to supplement widely consumed foods in such fatty acids. In this regard, poultry feeds enriched in synthetic CLA mixtures have emerged as another way of providing CLA to consumers.

Chamruspollert and Sell (1999) fed hens with 5% commercial CLA source, which consisted of 37% RmA, 47% *C18:2t10,cis12* and 16% other isomers of CLA. The egg yolks contained in percent of total fatty acids 5.97% RmA, 5.19% *C18:2t10,cis12* and 4.92% unidentified fatty acids, including other isomers of CLA. Cherian et al. (2002) reported that feeding hens with a diet supplemented with 2% synthetic CLA resulted in eggs containing 5.3% CLA of total yolk fatty acids, of which 3.4% RmA and 1.9% *C18:2t10,c12*. Several studies have indicated that these two isomers of CLA are metabolized in the body through different metabolic pathways and might therefore have different physiological consequences (Churruca, Fernandez-Quintela, & Portillo, 2009). A recent study described an increase in CLA in eggs from hens fed on pomegranate seed oil, indicating that the

conversion of PunA into RmA via Δ13 reductase activity is quite effective in this animal model. In this study, the CLA concentration in egg yolk lipids increased from 0.07 to 4.11% of fatty acids for the hens fed on a diet containing 1.5% PunA (Kostogrys et al., 2017).

Several studies have evaluated the effect of dietary CLA on the fatty acid profile of eggs from hens fed on n-3 PUFA-rich diets (Alvarez, Cachaldora, Méndez, GarcÍa-Rebollar, & De Blas, 2004; Du, Ahn, & Sell, 2000). In these conditions, the concentrations of CLA, saturated fatty acids (SFA) and long-chain n-3 PUFA increased in eggs, while those of long-chain n-6 polyunsaturated fatty acids (n-6 PUFA) decreased. A noticeable decrease could also be registered for monounsaturated fatty acids (MUFA), indicating an inhibition of the Δ9-desaturation activity. Regarding the impact of dietary CLnA, Kostogrys et al. (2017) also showed a decrease in MUFA in eggs, confirming an inhibitory effect already reported in rodent models (Arao et al., 2004; Shinohara et al., 2012). Furthermore, Yuan and colleagues reported that the content in linoleic acid and total n-6 PUFA was significantly reduced in the adipose tissue of mice fed on diets containing α-ESA and PunA, while the contents in DHA and total n-3 PUFA were significantly increased in the liver, kidneys and heart by PunA, but not by α-ESA (Yuan, Sinclair, Sun, & Li, 2009b). Thus, PunA, α-ESA and CLA may have differential effects on the activity of desaturases involved in the synthesis of n-6 and n-3 long-chain PUFA.

In the present study, we have investigated the effects of a combination of flaxseed with two different dietary sources of CLnA (*P. granatum* seed oil rich in PunA and *R. heudelotii* kernels or oil rich in α-ESA) in the diet of laying hens, on the fatty acid profile of their eggs. The main goal was to produce eggs naturally enriched in RmA and containing significant amounts of n-3 PUFA. A first experiment aimed at assessing the kinetics of RmA accumulation following supplementation with PunA or α-ESA. A second study was designed to assess the accumulation of RmA in the egg yolks from laying hens fed with increasing amounts of α-ESA, as well as

to determine to what extent the addition of α-ESA would affect the other fatty acids in egg yolk, with particular attention to the conversion of dietary ALA into DHA.

5.2. Materials and Methods

5.2.1. Ethics statement

This study was approved by the UCLouvain Animal Experiment Ethics Commission to ensure compliance with animal care and welfare guidelines. The experiments were conducted in one of the UCLouvain university farms (Corroy-le-Grand, Belgium). The hens were obtained from the Limal poultry farm (Wavre, Belgium). All efforts were made to minimize the number of hens in the trial, as well as to ensure their wellbeing.

5.2.2. Oils and plant seeds

Cold pressed unrefined pomegranate seed oil was purchased from Olvita (Pszenno, Poland). Extra virgin olive oil was obtained from local commercial sources. *R. heudelotii* kernels were obtained from local farmers in Cameroon. The kernels were crushed and the paste was pressed to extract oil.

5.2.3. Experimental design and diets

Trial 1: *Comparison of RmA accumulation in eggs by supplementing the diet of laying hens with α-ESA and PunA.*

Twelve 6-month-old red Sex-Link laying hens were individually housed in cages in a room maintained at about 18°C and received 14 hours of light/day. The trial started with a pre-experimental period of 10 days, during which the birds received a same diet enriched with flaxseeds (Dumoulin, Kortrijk, Belgium). Hens were then randomly assigned to three diets (4 hens/diet). The first treatment was the flaxseed enriched feed (FF). The two

other diets, FF-RHK and FF-PSO were formulated by adding to the staple feed FF, 10% (wt.) of ground *R. heudelotii* kernels (RHK) and 5% (wt.) of pomegranate seed oil (PSO), respectively. The addition of 5% pomegranate seed oil and 10% *R. heudelotii* kernels in the diets aimed at achieving equivalent fat levels in both experimental diets. Information concerning diets and fat sources is presented in Table 5.1. The experimental diets were administered for 12 days, after which all birds were fed again with FF for 10 days. Feed and water were supplied *ad libitum*. The egg harvesting started after the pre-experimental period, i.e. the first day of feeding with the experimental diets. Feed intake and egg production were recorded on a daily basis throughout the experiment. Eggs were collected daily, weighed whole and then broken. The yolks were separated from the other egg parts and weighed individually. The fatty acid composition, the cholesterol content as well as the total lipid content were determined on egg yolks.

Trial 2: *Accumulation of RmA in the yolks of laying hens fed with increasing amounts of α-ESA and influence of α-ESA hen diet supplementation on the n-3 PUFA profile of eggs.*

Twelve 6-month-old red Sex-Link laying hens were individually housed in cages under the same conditions as described previously, and were assigned to three experimental diets (4 hens/diets). The three diets were prepared from the flaxseed enriched feed (FF) used in Trial 1. Since the inclusion of *R. heudelotii* kernels slightly affected the particle size composition of the feed, the kernels were replaced with oil freshly prepared by cold pressing. The same amount of oil was added to the diets to obtain isolipidic diets. The first diet (FF-OL) contained feed enriched in flaxseed and 10% (wt.) of olive oil (OL). The second diet (FF-OL-RHO) contained the flaxseed enriched feed, 5% (wt.) of olive oil and 5% (wt.) of *R. heudelotii* kernel oil, and the last diet (FF-RHO) contained the flaxseed enriched feed and 10% (wt.) of *R. heudelotii* kernel oil (RHO). The diets were provided as follows: all

hens received FF-OL for 7 days to adapt the hens to a fatter diet and then were randomly assigned to the three diets. After the 21-day experimental feeding, all hens were fed again with FF-OL for 7 days. The experimental feeding period was longer (21 days) than in Trial 1 (12 days) in order to evaluate the persistence of RmA deposits in the lipids of the yolk. Feed and water were supplied *ad libitum*. Egg production, feed intake, egg and yolk masses were daily recorded throughout the experiment. The total lipid content, the cholesterol content and the fatty acid composition of the yolk were determined.

5.2.4. Extraction of total lipids

Total fat content was determined by the Soxhlet method (AOAC Official Method 925.32). An initial hydrolysis of the samples was performed in boiling hydrochloric acid in order to release the potentially bound fats from lipoprotein complexes and make them available for the extraction. The mixture was filtered so that all the fat was transferred to the filter and rinsed several times with distilled water until a neutral pH was obtained. The residues were dried and total (free and bound) fats were extracted with a classical Soxhlet device using diethyl ether (VWR Chemicals, Fontenay-sous-Bois, France) as solvent.

5.2.5. Analysis of fatty acid composition

Fatty acids from egg yolks were extracted following the Folch method (Folch, Lees, & Sloane Stanley, 1957). Tridecanoic acid (C13:0, Sigma-Aldrich) was used as internal standard in order to evaluate the efficiency of the extraction. Lipid extracts were dried under a continuous stream of nitrogen (N_2) and methylated as described earlier by Mellery et al. (2017). The resulting fatty acid methyl esters (FAME) were extracted with hexane (VWR Chemicals). FAME were separated and quantified by gas chromatography (GC).

The GC was equipped with an RT2560 capillary column (100 m × 0.25 mm, 0.2 µm film thickness, Restek), an automatic injector and a flame ionisation detector (FID) set at 255°C. The carrier gas used was H_2 at a constant pressure of 200 kPa. The oven temperature program was as follows: the initial temperature of the column was set at 80°C, it increased at 25°C/min up to 175°C, which was held during 25 min; a second increase at 10°C/min raised the column temperature up to 200°C; a holding temperature of 200°C lasted 20 min and was followed by an increase at 10°C/min up to 220°C; a holding temperature of 220°C lasted 5 min and was followed by an increase at 10°C/min up to 235°C, which was held during 15 min and followed by a decrease at 20°C/min to the initial temperature of 80°C. The chromatograms were analysed by the ChromQuest 5.0 software (ThermoFisher Scientific, Waltham Massachusetts, USA). Each peak was identified by comparison of retention times with those of pure methyl ester standards (Larodan, Solna, Sweden). The results were expressed in percentage (%) of the total identified fatty acids and in milligram per gram of egg yolk (mg/g of yolk).

5.2.6. Determination of cholesterol content

250 mg of dried egg yolk were saponified using 3 ml of potassium hydroxide dissolved in methanol (KOH/MeOH, 2M), with incubation in a shaking water bath at 50°C for 2 hours. The reaction was terminated by adding 1 ml of saturated sodium chloride (NaCl) solution, followed by cooling to room temperature. The unsaponifiable fraction was extracted using 2 ml of hexane, 3 times successively. The extract was then dried under N_2 and derivatized using a mixture of 99:1 N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), and anhydrous 99.8% pyridine (50:50, v/v) (Sigma-Aldrich) at 90°C for 30 min.

The analysis of the cholesterol trimethylsilyl (TMS) derivative was performed with a GC equipped with an RXi-5Sil MS column (30 m x 0.25 mm internal diameter x 0.25 µm film thickness, Restek), an on-column automatic injector and a flame ionisation detector. The injector and detector temperatures were set at 280°C and 305°C, respectively, and a splitless inlet was used to inject 1µl sample into the capillary column. The oven temperature was controlled with a ramp temperature program, which was initially set at 70°C and held for 4 min. The temperature was increased to 290°C at 30°C/min, and held for 30 min; then elevated at 320°C at the rate of 30°C/min and held for 5 min. Helium was the carrier gas at constant flow of 1.5 ml/min. Cholesterol was characterized by comparing its peak with a standard (Sigma-Aldrich).

5.2.7. Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and multiple-comparison tests on means using Tukey-Kramer HSD (honestly significant difference) test by JMP Pro version 13 (SAS, USA). The results were expressed as means and standard deviations. The differences between the means were considered significant at $p \leq 0.05$.

5.3. Results

5.3.1. Feed fatty acid profile, hen's feed consumption and egg production performance

Trials 1 and 2. The commercial diet (FF) had a total lipid content of 6.6% (wt.), whereas the experimental diets were much fattier (Table 5.1). Fatty acid profiles (in mg/g of feed) were also largely modified by the incorporation of the various lipid sources studied. In the supplemented diets of Trial 1, *R. heudelotii* kernels were included at 10% and pomegranate seed oil at 5% respectively,

leading to large increases in CLnA isomers and also in linoleic acid (*C18:2c9,cis12)* in the case of *R. heudelotii* kernel incorporation (Table 5.1). In Trial 2, *R. heudelotii* oil was included at 0, 5 or 10% in the diets. This led to a progressive decline in oleic acid (*C18:1cis9*), associated to a large increase in linoleic acid and α-ESA (Table 5.1).

Diet composition had a significant effect on the hen's feed consumption (Table 5.2). In Trial 1, lower consumptions were observed with the supplemented diets FF-RHK and FF-PSO. The feed intake was even lower for the dietary conditions of Trial 2, likely because of the higher fat in the diets used. The egg yield suggested some differences, but there was no significant effect. The weight of eggs showed some differences among the conditions tested in Trials 1 and 2. In Trial 1, the eggs obtained from the supplemented diets were heavier or similar to the control ones, while they were lighter or alike in Trial 2. The weight of egg yolk was slightly but significantly influenced by the inclusion of fat-rich ingredients in the diets. Indeed, it increased in the test conditions of Trial 1, while no significant difference could be registered in Trial 2. The cholesterol content in yolks was significantly different depending on the fat content of the diets. Egg yolks from hens fed the FF diet without fat supplementation in Trial 1 contained the lowest amount of cholesterol. There was no significant difference in the cholesterol content of the yolks in Trial 2, in which the same amount of fat was included in the diets. The different treatments did not significantly affect the total yolk fat content (in % of fresh yolk weight).

Table 5.1. Fat content and fatty acid composition of fat sources and experimental diets

	Fat sources				***Trial 1***			***Trial 2***		
	Flaxseed	OL	RHO	PSO	FF	FF-RHK	FF-PSO	FF-OL	FF-OL-RHO	FF-RHO
Total lipids (% of fresh material)	43.96	100	100	100	6.60	10.87	11.04	17.59	17.20	17.56
Fatty acids	% wt. of total identified fatty acids				mg/g of feed					
Palmitic acid (C16:0)	5.9	9.68	6.16	2.73	5.65	7.22	6.16	13.67	11.57	9.18
Stearic acid (C18:0)	4.21	3.17	6.77	1.94	2.22	3.98	2.68	4.57	5.52	6.47
Oleic acid (C18:1c9)	17.72	78.44	6.63	4.57	11.28	12.90	12.13	76.35	45.53	15.02
Cis-vaccenic acid (C18:1c11)	0.71	1.62	0.37	0.49	0.55	0.70	0.67	2.13	1.46	0.69
Linoleic acid (C18:2c9,c12)	14.90	4.82	30.07	5.21	21.52	28.31	21.07	24.30	33.04	39.10
Alpha-linolenic acid (C18:3c9,c12,c15)	55.35	0.71	0.06	0.84	14.76	13.58	13.21	12.68	12.40	12.77
Punicic acid (C18:3c9,t11,c13)	/	/	Traces	81.32	/	/	25.59	/	/	/
Alpha-eleostearic acid (C18:3c9,t11,t13)	/	/	48.62	Traces	/	10.18	/	/	14.25	29.51
Σ SFA	10.67	13.57	13.56	5.91	10.57	13.57	10.89	20.42	18.02	16.98
Σ MUFA	18.60	80.75	7.22	6.51	11.83	13.66	13.18	79.17	47.38	15.80
Σ n-6 PUFA	15.16	4.82	30.11	5.24	21.52	28.38	21.07	24.37	33.10	39.20
Σ n-3 PUFA	55.47	0.8	0.49	1.02	14.76	13.67	13.21	12.68	12.48	12.88

OL: Olive oil. RHO: *R. heudelotii* oil. PSO: Pomegranate seed oil. FF: Flaxseed-rich feed. FF-RHK: Flaxseed-rich feed supplemented with 10% *R. heudelotii* kernels. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil. FF-OL: Flaxseed-rich feed supplemented with 10% olive oil. FF-OL-RHO: Flaxseed-rich feed supplemented with 5% olive oil and 5% *R. heudelotii* oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil. SFA: Saturated fatty acids. MUFA: Monounsaturated fatty acids. n-6 PUFA: Omega-6 polyunsaturated fatty acids. n-3 PUFA: Omega-3 polyunsaturated fatty acids.

Table 5.2. Egg production parameters of hens subjected to the different diets: Feed intake, laying rate, weight of whole eggs and egg yolks, total lipid and cholesterol content

	Trial 1			***Trial 2***		
	FF	FF-RHK	FF-PSO	FF-OL	FF-OL-RHO	FF-RHO
Feed intake (g/hen/day)	$121.52^{a} \pm 37.37$	$112.57^{b} \pm 30.80$	$114.81^{b} \pm 24.54$	$99.19^{a} \pm 14.34$	$91.57^{b} \pm 18.33$	$98.30^{a} \pm 14.52$
Laying rate (%)	$85.71^{a} \pm 33.50$	$71.43^{a} \pm 29.16$	$91.07^{a} \pm 3.58$	$98.81^{a} \pm 1.52$	$97.37^{a} \pm 2.15$	$96.71^{a} \pm 4.98$
Whole eggs weights (g)	$59.11^{a} \pm 1.11$	$64.83^{b} \pm 4.08$	$59.42^{a} \pm 2.05$	$65.62^{a} \pm 6.26$	$62.60^{b} \pm 3.12$	$65.61^{a} \pm 7.27$
Yolks weights (g)	$14.31^{a} \pm 0.48$	$15.48^{b} \pm 1.13$	$15.28^{b} \pm 0.53$	$16.18^{a} \pm 1.42$	$15.90^{a} \pm 1.44$	$15.88^{a} \pm 1.75$
Yolk total lipids (% of fresh yolk weight)	$32.96^{a} \pm 2.31$	$32.57^{a} \pm 4.87$	$30.11^{a} \pm 2.25$	$31.10^{a} \pm 1.89$	$30.73^{a} \pm 2.20$	$31.22^{a} \pm 3.54$
Cholesterol (mg/fresh yolk)	$162.10^{a} \pm 33.86$	$184.41^{b} \pm 12.08$	$195.23^{b} \pm 11.82$	$188.25^{a} \pm 8.29$	$187.60^{a} \pm 19.50$	$185.04^{a} \pm 21.16$

Values (mean ± SD) in the same row within a trial with no common superscript letter (a, b, c) are significantly different at $p \leq 0.05$.
FF: Flaxseed-rich feed. FF-RHK: Flaxseed-rich feed supplemented with 10% *R. heudelotii* kernels. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil. FF-OL: Flaxseed-rich feed supplemented with 10% olive oil. FF-OL-RHO: Flaxseed-rich feed supplemented with 5% olive oil and 5% *R. heudelotii* oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil.

5.3.2. Kinetics of RmA, α-ESA and PunA accumulation in egg yolks

Trials 1 and 2. The evolution of RmA, α-ESA and PunA in egg yolks recorded during Trials 1 and 2, is shown in Figures 5.1 & 5.2 Before the administration of the oils, the RmA concentration of egg yolk was very low (< 0.05% wt. of total fatty acids) and neither α-ESA nor PunA was detected. Two days after administration of CLnA-oils, the concentration of RmA increased, whereas α-ESA and PunA became detectable in the yolks. RmA accumulation was gradual during the first days of feeding with CLnA sources. After the hens were returned to the control feed free of CLnA, the levels of RmA in eggs began to decrease. The same phenomenon was observed for CLnA, in particular for PunA whose accumulation had been greater in eggs.

Trial 1. The RmA levels in egg yolk showed a significant effect of treatment for *R. heudelotii* kernel and pomegranate seed oil feeding conditions (Figure 5.1.A). RmA levels (% wt. of total fatty acids) increased by 180-fold with the pomegranate seed oil supplemented diet and by 140-fold with the *R. heudelotii* kernel diet, as compared to the control. The α-ESA content remained very low in yolks throughout the feeding phase with the supplemented feed and did not exceed 1% of the total fatty acids. In contrast, PunA accumulated more importantly and reached 5% of the total fatty acids in yolk (Figure 5.1.B).

Trial 2. The concentration of RmA in the yolk lipids increased as the amount of *R. heudelotii* oil increased in the diet of hens. The RmA level in the yolks peaked 10 days after the start of the *R. heudelotii* oil feeding and remained constant until day 20 (FF-OL-RHO: $p = 0.1789$; FF-RHO: $p = 0.9210$) (Figure 5.2.A), after which the supplementation in the diet was stopped. During this plateau period obtained between the 10th and the 20th day of feeding, the RmA significantly differed ($p < 0.0001$) between the treatments (0, 5 and 10% (wt.) of *R. heudelotii* oil), rising from traces to, on average, 5.27 and 12.00% of the fatty acids in yolk, respectively.

The α-ESA content remained very low in the yolks and reached a maximum of 1.11% of the fatty acids of the yolk for the condition with 10% *R. heudelotii* oil (Figure 5.2.B).

5.3.3. Fatty acid composition of egg yolks

On the basis of the observations in Figure 5.2, the fatty acid compositions of yolks (in mg/g of yolk) presented in Table 5.3 are the average values of the results obtained for the eggs laid from day 10 to day 12 for Trial 1 and from day 10 to day 20 for Trial 2 (plateau periods).

Trial 1. The total SFA, i.e. the sum of lauric acid (*C12:0*), myristic acid (*C14:0*), palmitic acid (*C16:0*) and stearic acid (*C18:0*), in yolk did not vary significantly in response to dietary CLnA supplementation. As compared with the control group (FF), the hens fed on *R. heudelotii* kernels or pomegranate seed oil showed a decrease in total MUFA, and in each of their representatives, namely palmitoleic acid (*C16:1c9*), oleic acid (*C18:1c9*) and cis-vaccenic acid (*C18:1c11*), in egg yolk. Unlike its counterpart based on a pomegranate seed oil diet, the feeding with *R. heudelotii* kernels resulted in an increase in the level of linoleic acid in yolks. The two sources of CLnA did not influence the amount of arachidonic acid (*C20:4c5,c8,c11,c14*). Among the n-3 PUFA, the levels of ALA, docosapentaenoic acid (*C22:5c7,c10,c13,c16,c19*) and DHA in the lipids of yolk did not show statistically significant changes following the dietary supplementation of hens with CLnA. EPA accumulated in very small amounts in the yolks and its content was significantly lower in the treatment corresponding to the incorporation of *R. heudelotii* kernels in the feed. Of major interest, Table 5.3 shows a high increase of the concentration of RmA in yolk lipids from hens fed on *R. heudelotii* kernels or pomegranate seed oil, the greatest rise being observed with pomegranate seed oil. Neither α-ESA nor PunA were found in yolks from hens fed on the control diet. PunA concentration in the yolk was highly increased

upon hens' feeding on pomegranate seed oil, reaching levels up to 10 times higher than those found for α-ESA in the yolks obtained with the diet containing *R. heudelotii* kernels.

Trial 2. Replacing olive oil with *R. heudelotii* oil (5 or 10%) altered the proportions of SFA and MUFA in the lipids of egg yolk. The content in SFA, and more precisely in myristic, palmitic and stearic acids, increased significantly. Simultaneously, the levels of MUFA, namely palmitoleic, oleic and cis-vaccenic acids, decreased. The linoleic acid content was significantly higher in the lipids of the yolks of the conditions corresponding to the incorporation of *R. heudelotii* oil in the feed. Including *R. heudelotii* oil in the diet did however not affect the arachidonic acid concentration in yolks. The total n-3 PUFA in the yolk lipids linearly increased as the dietary CLnA source increased. The concentration of ALA increased in the yolks of hens fed with 5 and 10% *R. heudelotii* oil, in spite of the same dietary ALA content in all three diets. In contrast, the yolk DHA levels remained comparable for the three tested conditions and the amounts remained similar to those observed in Trial 1. The EPA levels remained very low, whatever the diet tested. The increase in *R. heudelotii* oil in the diet of hens resulted in a substantial increase in RmA concentration in yolks, leading to an average concentration of 34.51 mg/g of yolk for the FF-RHO condition. Considering an average yolk weight of 15.88 g, it means that each of these eggs contains 548 mg of RmA. The accumulation of α-ESA in the yolks was also influenced by the *R. heudelotii* oil content of the feed, but the concentrations in yolk remained at least 10 times more limited than those of RmA.

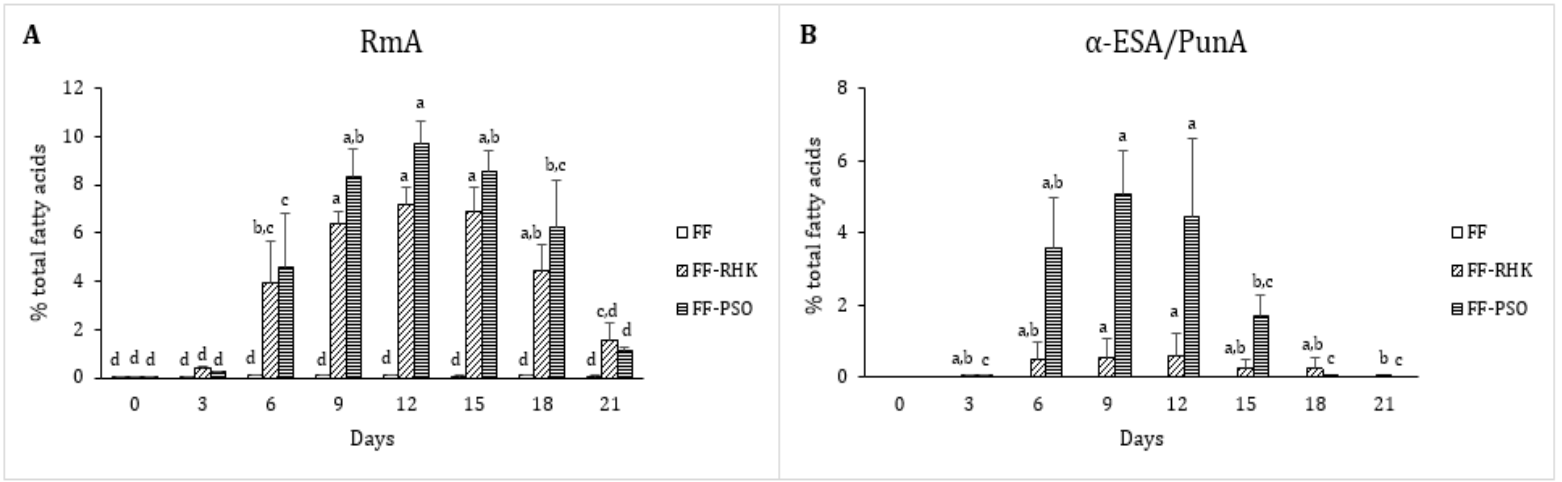

Figure 5.1. Evolution of the proportions (wt.% total fatty acids) of rumenic acid (RmA) (**A**), and α-eleostearic acid (α-ESA) or punicic acid (PunA) (**B**) in the egg yolks of hens fed for 12 days with diets supplemented with kernels of *R. heudelotii* or pomegranate seed oil, and then returned to the control diet (days 12 to 21). Values are means ± standard deviations. Mean values within the same diet with no common letter (a, b, c, d) are significantly different (ANOVA followed by Tukey's *post-hoc* test, α = 5%). FF: Flaxseed-rich feed. FF-RHK: Flaxseed-rich feed supplemented with 10% *R. heudelotii* kernels. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil.

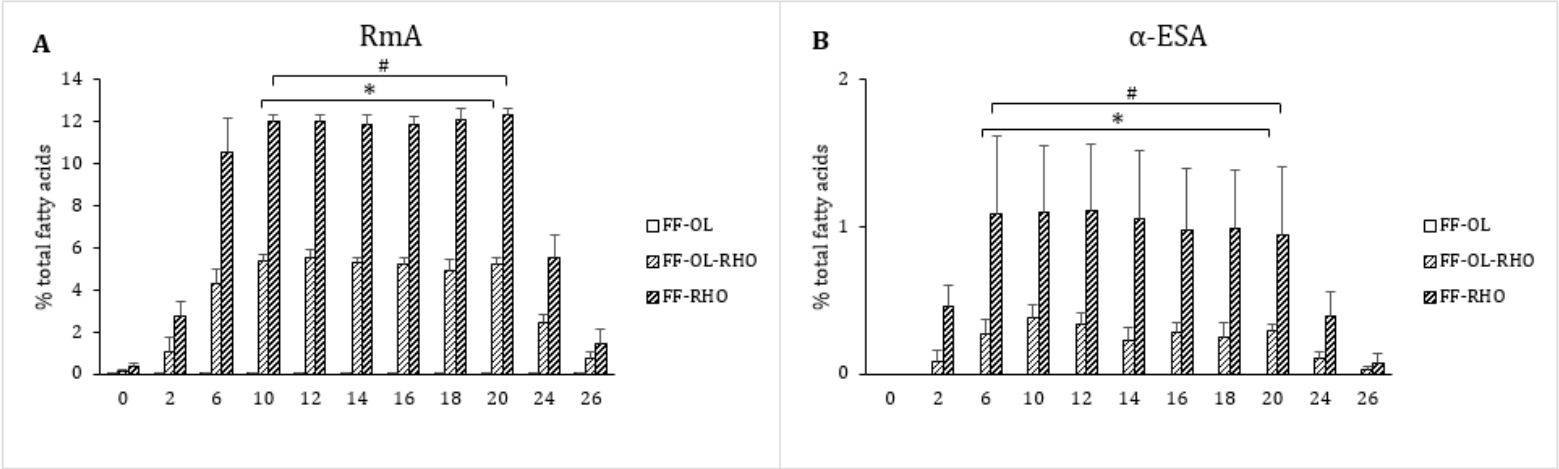

Figure 5.2. Evolution of the proportions (wt.% total fatty acids) of rumenic acid (RmA) (**A**) and α-eleostearic acid (α-ESA) (**B**) in the egg yolks of hens fed for 21 days with diets supplemented with *R. heudelotii* oil, and then returned to the control diet (days 21 to 26). Values are means ± standard deviations. (*) Mean values within the FF-OL-RHO diet are not significantly different during this period. (#) Mean values within the FF-RHO diet are not significantly different during this period (ANOVA, α = 5%). FF-OL: Flaxseed-rich feed supplemented with 10% olive oil. FF-OL-RHO: Flaxseed-rich feed supplemented with 5% olive oil and 5% *R. heudelotii* oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil.

Table 5.3. Effect of diets on fatty acid composition of egg yolks

Fatty acids (mg/g of yolk)	***Trial 1***			***Trial 2***		
	FF *n*=8	FF-RHK *n*=8	FF-PSO *n*=7	FF-OL *n*=23	FF-OL-RHO *n*=22	FF-RHO *n*=23
Lauric acid (C12:0)	$0.25^{a} \pm 0.05$	$0.22^{a} \pm 0.09$	$0.19^{a} \pm 0.03$	$0.22^{a} \pm 0.10$	$0.21^{a} \pm 0.03$	$0.26^{a} \pm 0.26$
Myristic acid (C14:0)	$0.81^{a} \pm 0.21$	$0.79^{a} \pm 0.03$	$0.81^{a} \pm 0.18$	$0.47^{a} \pm 0.06$	$0.62^{b} \pm 0.05$	$0.74^{c} \pm 0.10$
Palmitic acid (C16:0)	$57.35^{a} \pm 13.81$	$59.08^{a} \pm 7.64$	$61.25^{a} \pm 3.82$	$49.72^{a} \pm 8.45$	$56.73^{b} \pm 4.71$	$61.74^{c} \pm 6.01$
Palmitoleic acid (C16:1c9)	$7.16^{a} \pm 2.97$	$4.10^{b} \pm 0.43$	$3.66^{b} \pm 0.24$	$4.95^{a} \pm 0.88$	$2.79^{b} \pm 0.31$	$2.14^{c} \pm 0.26$
Stearic acid (C18:0)	$20.98^{a} \pm 3.51$	$23.48^{a} \pm 3.13$	$24.54^{a} \pm 1.55$	$19.40^{a} \pm 3.55$	$22.23^{b} \pm 2.47$	$27.91^{c} \pm 2.62$
Oleic acid (C18:1c9)	$84.92^{a} \pm 22.03$	$65.05^{b} \pm 7.76$	$66.29^{b} \pm 3.08$	$132.94^{a} \pm 21.60$	$102.97^{b} \pm 9.06$	$64.65^{c} \pm 7.33$
Cis-vaccenic acid (C18:1c11)	$4.27^{a} \pm 1.19$	$2.74^{b} \pm 0.31$	$2.63^{b} \pm 0.09$	$4.12^{a} \pm 1.60$	$2.92^{b} \pm 0.30$	$2.02^{c} \pm 0.12$
Linoleic acid (C18:2c9,c12)	$39.49^{a,b} \pm 10.74$	$45.95^{a} \pm 7.75$	$36.48^{b} \pm 2.39$	$29.99^{a} \pm 4.82$	$43.45^{b} \pm 4.33$	$61.14^{c} \pm 5.30$
Alpha-linolenic acid (C18:3c9,c12,c15)	$13.54^{a} \pm 4.33$	$10.66^{a} \pm 1.95$	$11.52^{a} \pm 1.11$	$5.88^{a} \pm 0.67$	$7.95^{b} \pm 0.91$	$10.51^{c} \pm 1.42$
Rumenic acid (C18:2c9,t11)	$0.19^{a} \pm 0.06$	$16.51^{b} \pm 3.21$	$23.51^{c} \pm 3.73$	$0.07^{a} \pm 0.04$	$14.52^{b} \pm 1.55$	$34.51^{c} \pm 3.48$
Punicic acid (C18:3c9,t11,c13) / alpha-eleostearic acid (C18:3c9,t11,t13)	ND^{a}	$1.36^{a} \pm 0.39$	$13.70^{b} \pm 2.86$	ND^{a}	$0.88^{b} \pm 0.26$	$3.01^{c} \pm 1.14$
Arachidonic acid (C20:4c5,c8,c11,c14)	$3.00^{a} \pm 0.49$	$3.01^{a} \pm 0.49$	$3.12^{a} \pm 0.26$	$3.56^{a} \pm 0.43$	$3.56^{a} \pm 0.34$	$3.58^{a} \pm 0.36$
Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)	$0.34^{a} \pm 0.07$	$0.19^{b} \pm 0.03$	$0.34^{a} \pm 0.07$	$0.17^{a} \pm 0.06$	$0.14^{a,b} \pm 0.03$	$0.11^{b} \pm 0.04$
n-3 Docosapentaenoic acid (C22:5c7,c10,c13,c16,c19)	$1.09^{a} \pm 0.24$	$0.79^{a} \pm 0.11$	$0.92^{a} \pm 0.24$	$0.72^{a} \pm 0.11$	$0.97^{b} \pm 0.45$	$0.90^{a,b} \pm 0.15$
Docosahexaenoic acid (C22:6c4,c7,c10,c13,c16,c19)	$5.34^{a} \pm 0.70$	$5.01^{a} \pm 0.72$	$5.86^{a} \pm 0.53$	$5.55^{a} \pm 0.77$	$5.22^{a} \pm 0.68$	$4.95^{a} \pm 0.55$

Table 5.3. Effect of diets on fatty acid composition of egg yolks (continued)

Fatty acids (mg/g of yolk)	***Trial 1***			***Trial 2***		
	FF *n*=8	FF-RHK *n*=8	FF-PSO *n*=7	FF-OL *n*=23	FF-OL-RHO *n*=22	FF-RHO *n*=23
Σ SFA	$81.33^{a} \pm 17.72$	$85.75^{a} \pm 10.17$	$89.24^{a} \pm 5.27$	$73.74^{a} \pm 9.99$	$81.86^{b} \pm 6.75$	$93.35^{c} \pm 8.16$
Σ MUFA	$97.20^{a} \pm 25.50$	$72.76^{b} \pm 8.72$	$73.61^{b} \pm 3.17$	$148.37^{a} \pm 18.55$	$112.23^{b} \pm 9.85$	$71.27^{c} \pm 8.11$
Σ n-6 PUFA	$43.71^{a,b} \pm 11.06$	$50.65^{a} \pm 8.38$	$41.09^{b} \pm 2.64$	$34.43^{a} \pm 5.25$	$48.38^{b} \pm 4.71$	$66.55^{c} \pm 5.76$
Σ n-3 PUFA	$22.36^{a} \pm 5.86$	$16.88^{b} \pm 2.75$	$18.76^{a,b} \pm 1.23$	$12.44^{a} \pm 1.49$	$14.36^{b} \pm 1.84$	$16.67^{c} \pm 1.73$

Values (mean ± SD) in the same row within a trial with no common superscript letter (a, b, c) are significantly different at $p \leq 0.05$.
n: number of eggs from day 10 to day 12 (Trial 1) and from day 10 to day 20 (Trial 2). FF: Flaxseed-rich feed. FF-RHK: Flaxseed-rich feed supplemented with 10% *R. heudelotii* kernels. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil. FF-OL: Flaxseed-rich feed supplemented with 10% olive oil. FF-OL-RHO: Flaxseed-rich feed supplemented with 5% olive oil and 5% *R. heudelotii* oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil. ND: Not detected. SFA: Saturated fatty acids. MUFA: Monounsaturated fatty acids. n-6 PUFA: Omega-6 polyunsaturated fatty acids. n-3 PUFA: Omega-3 polyunsaturated fatty acids.

5.4. Discussion

Feeding synthetic CLA oil has been the most widely used and documented method for enriching eggs with CLA. The discovery of CLnA and the evidence that they could be converted into CLA have allowed the development of more natural enrichment methods (Kostogrys et al., 2017; Lee et al., 2002). However, the dietary use of some CLnA-rich oils has been found to induce some toxic effects on hens. Crude tung oil, an oil rich in α-ESA, added at 2% wt. in the ration of laying hens caused a sharp drop in egg production rate to 30% in 10 days, and higher inclusion (8% wt.) reduced egg production to less than 5%. Other adverse effects reported include an abrupt drop in body weight, diarrhea, loss of coloration and reduction in size of comb and wattles, loss of feathers and severe convulsions (Edwards, Suso, & Mason, 1967). The challenge of the strategy of natural enrichment of eggs with CLA using CLnA sources would be to find a food naturally rich in CLnA, which even at high doses would not have harmful effects on health. In that respect, pomegranate seed oil has recently been used to feed laying hens, at an inclusion rate of up to 2.5% wt. in diets, without any toxic effect (Kostogrys et al., 2017). In the present study, pomegranate seed oil was used at a higher inclusion rate (5% wt.) without any noticeable negative effect on production (rate of egg production, weight of eggs and yolks). In addition, the α-ESA-rich *R. heudelotti* oil has been used here, for the first time, as an alternative dietary source of CLnA to enrich eggs with RmA. No toxic effect could be registered, which was expected since the seeds of this plant are commonly used by people in Western and Central Africa as ingredients in local dishes.

Globally, the CLnA-supplemented diets did not influence the total fat content of yolk, which is in agreement with other studies indicating that the overall lipid content of the yolk is not altered by the inclusion of CLnA sources in hen's feed (Kostogrys et al., 2017; Lee et al., 2002). Since egg yolk lipids are intended for use as an energy source by the embryo, the fact that their amount can be

materially influenced by diet could represent a considerable risk for embryonic development. It therefore seems possible that during the synthesis of the yolk, the hen exerts a regulatory control, and there may be an overall lipid content, which is stable (30 to 33% of the wet mass of yolk) and cannot be changed by feeding. However, the cholesterol content of the yolk may increase in response to a high fat content in the diet.

An increase in RmA content was observed in the eggs of all the groups fed with CLnA. After 10 days of α-ESA or PunA feeding, RmA reached its maximum concentration in the yolk. A significant part of the dietary lipids ingested by the laying hen is used for the synthesis of yolk, thus acting on the vitellogenesis and the composition of lipid deposits in the oocyte. In hens, the rapid growth phase prior to ovulation for the yolk to develop and fill the mature oocyte is 7 to 11 days. During this period, the yolk increases in size and rapidly accumulates a large quantity of lipids synthesized by the liver. The order in which the oocytes are released corresponds to the order of their maturation and results from their sequential development. Nys and Guyot (2011) noted that approximately 98% of the egg yolk is deposited linearly in the oocyte throughout the rapid growth phase.

The conversion of α-ESA into RmA appeared to be higher than that of PunA, which was more accumulated in egg yolk. In Trial 1, larger amounts of RmA were found in the yolk with the pomegranate seed oil diet FF-PSO, but this was due to its higher content in PunA (25.59 mg/g of feed) compared to that in α-ESA (10.18 mg/g of feed) in the *R. heudelotii* kernels' diet FF-RHK. In Trial 2, the highest α-ESA diet (FF-RHO, 29.51 mg/g of feed) resulted in an extremely high amount of RmA in the yolk, while the accumulation of α-ESA remained low in the yolk (less than 2% of fatty acids), indicating that a large part of ESA has been converted to RmA. This result is in agreement with those of Yuan et al. (2009c) and Schneider et al. (2013) who reported respectively in mice and Caco-2 cells, that the difference in conversion efficiency would be

due to the geometric configuration of the Δ13 double bond. Indeed, α-ESA possesses a *trans* Δ13 double bond, whereas PunA has a *cis* Δ13 double bond. The geometric structure would affect the activity of the enzyme involved in the Δ13-double bond saturation, hence the difference in conversion rate of both substrates. It appears that this enzyme would have a greater affinity for the double bond in its *trans* configuration. Eggs produced by hens fed with 10% *R. heudelotii* oil (Trial 2) could each provide 548 mg of RmA and 48 mg of α-ESA, along with 265 mg of n-3 PUFA. Such a high concentration of RmA in eggs has never been reached before, neither by supplementation with synthetic CLA, nor with oils rich in CLnA. To date, the maximum levels of incorporation into the egg yolk have not exceeded 365 mg of CLA per egg (Chamruspollert & Sell, 1999). The enrichment of egg lipids in RmA was CLnA dose-dependent (Trial 2). The substantial level of RmA incorporated into egg yolk lipids may be due to the high proportion of CLnA-rich oils in the diet fed to hens.

The overall changes in SFA and MUFA content of yolk observed in the current research are in line with those reported by Kostogrys et al. (2017). These authors found a significant increase in the amount of SFA with a concomitant reduction in MUFA in egg yolks following the addition of pomegranate seed oil in hen's feed. Several studies have indicated that CLnA inhibit Δ9-desaturation in the liver through inhibition of the stearoyl-CoA desaturase (SCD) activity, and the reduction of the SCD-1 mRNA expression (Arao et al., 2004; Shinohara et al., 2012). This enzyme catalyses the Δ9-*cis* desaturation of fatty acyl-CoA substrates and converts palmitoyl- and stearoyl-CoA, into palmitoleoyl- and oleoyl-CoA, respectively. An inhibitory effect of RmA on SCD activity has also been reported by Choi et al. (2002). In the present study, it cannot be clearly established whether the inhibition of SCD is caused by the individual action of either α-ESA or PunA, or either RmA, or by the synergistic action of RmA with its precursors. Moreover, n-6 and n-3 PUFA have been reported to repress the expression of the SCD-1

gene in the liver, adipose tissue, and other tissues such as the brain and immune tissues (Ntambi, 1999). SCD has proven to be the critical point in regulating the flow of endogenous and dietary fatty acids in metabolically active or storage pools. By determining the cellular SFA/MUFA balance, it also has an important effect on the fluidity of the membrane and thus influences many biological processes and systems. Studies have shown that SCD is overexpressed in many types of cancer and that SCD-1 activity increases MUFA levels in cancer cells to ensure their survival and proliferation. As a result, inhibition of SCD-1 activates apoptosis and exposes cancer cells to the cytotoxic effects of SFA (Scaglia & Igal, 2005). Several reports have claimed that SCD inhibitors, such as pyridin-2-ylpiperazine-based compounds and sterculic acid have therapeutic effects against several types of cancer, and against diet-induced obesity and other diseases associated to the metabolic syndrome (Peláez, Pariente, Pérez-Sala, & Larráyoz, 2020; Zhang et al., 2013). However, potentially harmful consequences of SCD-1 inhibition can occur following the accumulation of SFA in tissues. Indeed, SFA accumulation may promote inflammatory diseases including atherosclerosis and steatohepatitis (Gentile & Pagliassotti, 2008; Temel & Rudel, 2007). The pro-inflammatory effects resulting from the accumulation of SFA can be counteracted by dietary supplementation with n-3 PUFA (Brown et al., 2010). Given their known health benefits, it is reasonable to speculate that α-ESA or PunA and/or RmA could be used for therapeutic purposes as inhibitors of SCD-1, and administered in conjunction with n-3 PUFA, to avoid potential deleterious side effects of SFA. Additional work is necessary to formally attest this opportunity.

Belury and Kempa-Steczko (1997) reported the ability of CLA to compete with linoleic acid or ALA for Δ6 desaturase, the rate-limiting step in the conversion of these fatty acids to, respectively, arachidonic acid or EPA and DHA, in the liver of mice. They found that CLA were converted by Δ6 desaturase to unidentified octadecatrienoic acids to an extent similar to the

conversion of linoleic acid to γ-linolenic acid (*C18:3c6,c9,c12*). Accordingly, Banni (2002) indicated that CLA isomers undergo desaturation and elongation processes similar to those occurring with linoleic acid while maintaining the conjugated diene structure. In the present study, the content in arachidonic acid and DHA remained stable in the lipids of the yolk, while a slight decrease was registered for EPA, at least in Trial 2, suggesting only a limited effect of the competition generated by RmA. In contrast, as RmA increased in Trial 2, more ALA accumulated in yolk lipids, whereas its content was stable in the feed, suggesting that it was less used as energy source by the hen. On the other hand, the accumulation of linoleic acid in the yolks was related to its concentration in the diets of Trial 2, *R. heudelotii* oil being rich in this fatty acid.

5.5. Conclusion

The present study aimed at developing, in natural way, new eggs with a high content in ALA, DHA, RmA, and to lesser extent α-ESA or PunA, to provide consumers with eggs with a specific health potential. This is the first investigation aiming to combine these fatty acids in egg yolks. The two different CLnA sources (*R. heudelotii* and pomegranate seed oils) supplemented in the hen's diet allowed a considerable increase in the RmA content to over 500 mg/egg. Eating a single of those eggs per day would already cover about 18% of the estimated needs allowing to reach beneficial effects of dietary RmA. Further studies are needed to determine a potential impact of the long-term feeding strategy on the composition of the eggs and the health of hens. Analyses on the physico-chemical characteristics of eggs, as well as their sensory properties and other quality parameters such as shelf life and physico-chemical changes during storage, which are important for consumer acceptance, are also needed.

5.6. References

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5.7. Additional data

Table 5.4. Average fatty acid composition of standard eggs and eggs enriched with flaxseed, whether or not combined with pomegranate seed oil or *R. heudelotii* oil

Fatty acids (mg/egg)	**Standard**	**FF**	**FF-PSO**	**FF-RHO**
Lauric acid (C12:0)	1.92 ± 0.17	3.58 ± 0.72	2.90 ± 0.46	4.13 ± 3.89
Myristic acid (C14:0)	13.46 ± 1.50	11.59 ± 3.01	12.38 ± 2.75	11.75 ± 1.59
Palmitic acid (C16:0)	908.01 ± 44.28	820.68 ± 197.62	935.90 ± 58.37	980.43 ± 95.44
Palmitoleic acid (C16:1c9)	116.02 ± 16.65	102.46 ± 42.50	55.92 ± 3.67	33.98 ± 4.13
Stearic acid (C18:0)	234.07 ± 17.39	300.22 ± 50.23	374.97 ± 23.68	443.21 ± 41.60
Oleic acid (C18:1c9)	1412.39 ± 87.91	1215.20 ± 315.25	1012.91 ± 47.06	1026.64 ± 116.40
Cis-vaccenic acid (C18:1c11)	79.04 ± 10.67	61.10 ± 17.03	40.19 ± 1.37	32.08 ± 1.90
Linoleic acid (C18:2c9,c12)	502.16 ± 25.68	565.10 ± 153.69	557.41 ± 36.52	970.90 ± 84.16
Alpha-linolenic acid (C18:3c9,c12,c15)	11.44 ± 0.86	193.76 ± 61.96	176.02 ± 16.96	166.90 ± 22.55
Rumenic acid (C18:2c9,t11)	2.04 ± 0.32	2.72 ± 0.86	359.23 ± 56.99	548.02 ± 55.26
Punicic acid (C18:3c9,t11,c13) / alpha-eleostearic acid (C18:3c9,t11,t13)	ND	ND	209.34 ± 43.70	47.80 ± 18.10
Arachidonic acid (C20:4c5,c8,c11,c14)	63.65 ± 10.35	42.93 ± 7.01	47.67 ± 3.97	56.85 ± 5.72
Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)	ND	4.86 ± 1.00	5.19 ± 1.07	1.75 ± 0.63
n-3 Docosapentaenoic acid (C22:5c7,c10,c13,c16,c19)	3.13 ± 0.50	15.60 ± 3.43	14.06 ± 3.67	14.29 ± 2.38
Docosahexaenoic acid (C22:6c4,c7,c10,c13,c16,c19)	20.01 ± 5.84	76.41 ± 10.02	89.54 ± 8.10	78.61 ± 8.73

Table 5.4. Average fatty acid composition of standard eggs and eggs enriched with flaxseed, whether or not combined with pomegranate seed oil or *R. heudelotii* oil (continued)

Fatty acids (mg/egg)	**Standard**	**FF**	**FF-PSO**	**FF-RHO**
Σ SFA	1176.98 ± 62.08	1163.83 ± 253.57	1363.59 ± 80.52	1482.40 ± 129.58
Σ MUFA	1626.18 ± 113.93	1390.93 ± 364.90	1124.76 ± 48.44	1131.77 ± 128.79
Σ n-6 PUFA	598.24 ± 34.91	625.49 ± 158.27	627.85 ± 40.34	1056.81 ± 91.47
Σ n-3 PUFA	35.02 ± 5.64	319.97 ± 83.86	286.65 ± 18.79	264.72 ± 27.47

FF: Flaxseed-rich feed. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil. SFA: saturated fatty acids. MUFA: monounsaturated fatty acids. n-6 PUFA: omega-6 polyunsaturated fatty acids. n-3 PUFA: omega-3 polyunsaturated fatty acids. ND: Not detected.

Table 5.5. Composition of laying hens' diets

In percentage (%) of feed	**FF diet** [a]	**FF-PSO diet**	**FF-RHO diet**	**Nutritional recommendations** [b]
Crude protein	16.7	15.9	15.0	max. 20
Fat	6.7	11.0	17.6	4 – 7
Crude fiber	2.6	2.5	2.3	max. 7
Calcium	4.5	4.3	4.1	min. 3.5
Phosphorus	0.52	0.50	0.47	min. 0.31
Sodium	0.13	0.12	0.12	min. 0.12
Lysine	0.85	0.81	0.77	min. 0.65
Methionine	0.41	0.39	0.37	min. 0.3

FF: Flaxseed-rich feed. FF-PSO: Flaxseed-rich feed supplemented with 5% pomegranate seed oil. FF-RHO: Flaxseed-rich feed supplemented with 10% *R. heudelotii* oil.

[a] Ingredients: corn, wheat, soy meal, calcium carbonate, flaxseed oil, monocalcium phosphate, sodium chloride, sodium bicarbonate.
Additives/kg: Vitamin A 10,000 IU; Vitamin D3 1500 IU; Vitamin E 100 mg; Ferrous sulfate, monohydrate, 55 mg; calcium iodate 1.80 mg; Cupric sulfate, pentahydrate, 20 mg; Manganous oxide, 75 mg; Zinc sulphate, monohydrate, 70 mg; Sodium selenite, 0.40 mg; Lutein 2.3 mg; Canthaxanthin 3.5 mg; Zeaxanthin 0.11 mg.

[b] Ref.: Leroyer et al. Cahier technique : Produire des œufs biologiques en AB. ITAB, 2010.

Chapter 6

The egg yolk content in ω-3 and conjugated fatty acids can be sustainably increased upon long-term feeding of laying hens with a diet containing flaxseeds and pomegranate seed oil

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Foreword

Following on from Chapter 5, this work implemented the nutritional strategy developed to enrich eggs with omega-3 fatty acids, rumenic acid and punicic acid in a hen-rearing activity. At the same time, some issues were addressed, including the distribution of fatty acids in egg yolk lipids and changes in fatty acid profiles of hen tissues.

Abstract

Long-term feeding trials examining the incorporation of conjugated linolenic acids (CLnA) into the diet of laying hens are lacking. In the present study, we compared two diets in sixty-six red Sex-Link hens (33 hens/treatment), fed for 26 weeks. The control diet was high in oleic acid, while the test diet was high in α-linolenic acid (ALA) and punicic acid (PunA). No significant differences were observed between treatments for hens' performance, egg weight and yolk weight. In contrast, dietary ALA and PunA resulted in a significant increase in n-3 PUFA, rumenic acid (RmA) and PunA contents in egg yolk, as well as in the liver, heart, muscle and adipose tissue of the hens. Other conjugated dienes resulting from the metabolism of PunA or RmA also accumulated in the egg yolk and tissues. Unlike DHA, which was exclusively distributed in phospholipids, ALA, RmA and PunA were preferably distributed in triglycerides.

Keywords: Conjugated fatty acids; Egg yolk; Flaxseed; Pomegranate seed oil; Alpha-linolenic acid; Docosahexaenoic acid; Punicic acid; Rumenic acid.

6.1. Introduction

One of the fascinating findings in human nutrition is that certain foods, beyond providing essential nutrients to the body, contain high concentrations of biologically active compounds that are effective in preventing or treating chronic or non-communicable diseases, among which cardiovascular diseases, diabetes and cancers (Roberfroid, 2011; Siró, Kápolna, Kápolna, & Lugasi, 2008). As a result, nutritional strategies have been extensively developed to enrich widely consumed foods with such beneficial compounds, in order to increase their dietary intake without drastic changes in diet or eating habits. In this sense,

chicken eggs have been found as an excellent candidate for nutritional enrichment.

As one of the cheapest sources of animal protein and fat consumed all over the world, eggs are a significant component of the human diet. Moreover, by modifying the feed of laying hens it is possible to significantly increase the levels of certain nutrients in the egg to such an extent that consuming a single egg could provide these nutrients in relevant amounts, as compared to the recommended daily allowance (Surai & Sparks, 2001). For example, plant seeds rich in α-linolenic acid (ALA, *C18:3c9,c12,c15*), such as flaxseeds, have been commonly added to hens' diet to enrich eggs mainly with ALA and also, to some extent, with one of its long-chain anabolic derivative, docosahexaenoic acid (DHA, *C22:6c4,c7,c10,c13,c16,c19*). Omega-3 polyunsaturated fatty acids (n-3 PUFA) have shown health benefits in terms of inflammatory processes (Calder, 2017; Reifen, Karlinsky, Stark, Berkovich, & Nyska, 2015) and neurodegenerative diseases (Avallone, Vitale, & Bertolotti, 2019). Long-chain n-3 PUFA but not ALA have been associated with a low risk of cardiovascular diseases (Bowen, Harris, & Kris-Etherton, 2016; Harris et al., 2021). In addition, DHA is known to be of utmost importance for the proper development and functioning of the nervous system (Brenna & Carlson, 2014). Conjugated linoleic acids (CLA) and conjugated linolenic acids (CLnA) have been shown to have a wide range of biological activities, among which anti-diabetic (Moloney, Yeow, Mullen, Nolan, & Roche, 2004; Nekooeian, Eftekhari, Adibi, & Rajaeifard, 2014), anti-inflammatory (Riserus, Vessby, Arnlov, & Basu, 2004) and anti-cancer (Dhar Dubey, Sharma, & Kumar, 2019; Sailas & Friedrich, 2009) properties. The bulk of CLA is supplied to humans by the diet, especially through the consumption of dairy products and ruminant meat (Chin, Liu, Storkson, Ha, & Pariza, 1992), but dietary intake remains relatively low as CLA account for less than 2% of total ruminant fat. On the other hand, CLnA are present in large amounts in the oil of certain plant seeds, such as

pomegranate, ricinodendron, bitter gourd or catalpa (Hennessy, Ross, Devery, & Stanton, 2011). A Δ13-reductase activity allowing the conversion of certain CLnA into CLA has been found in animal and human tissues (Schneider, Mignolet, Schneider, & Larondelle, 2013; Tsuzuki et al., 2004; Yuan, Sinclair, Xu, & Li, 2009). We have previously shown that dietary sources of CLnA such as pomegranate seed oil (PSO), high in punicic acid (PunA, *C18:3c9,t11,c13*), supplemented in the hen's diet allowed a considerable increase in the egg content of PunA, as well as rumenic acid (RmA, *C18:2c9,t11*), an omega-7 CLA (Ngo Njembe et al., 2021a). Thus, feeding laying hens with diets containing CLnA is a means for incorporating both CLnA and CLA into the egg yolk fat.

Yet, there have been few studies on CLnA dietary supplementation in laying hens, and those reported were short-term feeding trials (less than 3-month long). In addition, they did not address the fate of CLnA in hens' tissues. However, studies in rodents have indicated that CLnA are incorporated in animal tissues as CLA, and the predominant form is RmA (Arao et al., 2004; de Melo et al., 2016). Besides, this accumulation of RmA goes along with a decrease in total omega-6 (n-6) PUFA in heart and adipose tissue and an increase in total n-3 PUFA in the liver and heart (Yuan, Sinclair, Sun, & Li, 2009). This suggests that feeding the hens with PunA might also alter the fatty acid profile of their tissues. Therefore, understanding the effects of a CLnA-rich diet on the fatty acid composition of hens' tissues is an important consideration in designing an effective enrichment program.

In a previous study, we reported the effect of the dietary combination of flaxseeds and PSO on the fatty acid profile of eggs during a short period of the laying cycle (12 to 21 days) using a reduced number of individually housed hens (4 hens/diet) (Ngo Njembe et al., 2021a). For the current study, the objective was to determine the performance of the hens, and the incorporation of fatty acids in the egg yolk over a fairly long production period (26 weeks) under regular conditions of an egg production unit, with a

larger number of hens housed in the same pen. In the current assessment, we examine the interaction between the length of the feeding period and the levels of ALA, DHA, RmA and PunA in eggs, as well as the distribution of these fatty acids into lipid classes. We also report on the presence of conjugated dienes resulting from the metabolism of PunA and/or RmA in egg yolk. In addition, we determine how a 26-week long supplementation with dietary flaxseeds and PSO affects the fatty acid composition of different hens' tissues.

6.2. Materials and Methods

6.2.1. Birds, housing and diets

Sixty-six 5-month-old red Sex-Link hens obtained from the Limal poultry farm (Wavre, Belgium) were housed in floor pens maintained under controlled temperature and lighting conditions as described previously (Ngo Njembe et al., 2021a), and fed with a standard feed for egg-producing hens (Aveve, Leuven, Belgium). At 7 months of age, the hens were randomly divided into two separate lots, and assigned to two different diets (33 hens/diet). One group was fed the standard feed supplemented with 10% (wt.) extra virgin olive oil obtained from a local commercial source (F-OL diet). The other group received the standard feed added with 7.8% (wt.) flaxseeds (Dumoulin, Kortrijk, Belgium) and 7% (wt.) PSO (Ol'vita, Pszenno, Poland) (FS-PSO diet). The diets were formulated to differ in fatty acid composition and contained a high amount of either oleic acid (OA, *C18:1c9*) (F-OL diet) or ALA and PunA (FS-PSO diet). The inclusion rates for olive oil and the mix of flaxseeds and PSO were made up to equalize the total fat content of each diet at 15%. Fresh diets were prepared daily to minimize oxidation of the fats. Feed samples from each diet were taken after mixing, and analysed as described later. The fatty acid composition of the diets is shown in Table 6.1. The dietary treatments were administered for 26 weeks, and all animals had free access to food and water.

6.2.2. Sample collection and measurements

Eggs were harvested daily throughout the study from fourteen days after the start of the experimental feeding to allow substantial deposition of fatty acids in the egg yolk. Egg production was calculated from the total of eggs divided by the number of days and hens, and expressed as average hen's production per day. The fatty acid composition of the eggs was determined at weekly interval until the end of the trial from six eggs randomly selected in each treatment. The eggs were weighed whole and then cracked. The yolks were separated from the albumen and the shell, and weighed individually. Results of four consecutive weeks were grouped to build up 6 periods of 28 days each. At the end of the feeding period, five birds from each treatment group were randomly selected and euthanized by exsanguination. Liver, heart, adipose tissue and abdominal muscles were collected and kept frozen at -20°C for further fatty acid analysis.

6.2.3. Lipid extraction and fatty acid analysis

The fatty acid composition of feeds, hens' tissues and egg yolks was evaluated after chloroform-methanol-water extraction of the lipids from the samples. The Folch method (Folch, Lees, & Sloane Stanley, 1957) was used for feed and egg yolk samples, and tissue lipids were extracted by the Bligh & Dyer method (Bligh & Dyer, 1959). Lipids extracted from tissues and some yolk samples were separated by solid phase extraction (SPE) into three lipid fractions: triglycerides (TG), free fatty acids (FFA) and phospholipids (PL), using a Bond Elut–NH_2, 200 mg, 3 ml silica-based SPE column (Agilent technologies, Santa Clara, CA, USA), as described previously (Schneider et al., 2013). Fatty acids (extracted from total lipids and lipid fractions) were converted into fatty acid methyl esters (FAME) via methylation under alkaline conditions (KOH/MeOH, 0.1 M, at 70°C for 60 min) and then under acidic conditions (HCl/MeOH, 1.2 M, at 70°C for 20 min). The resultant

FAME were subsequently extracted using hexane (VWR Chemicals, Radnor, PA, USA) and separated by gas chromatography (GC).

6.2.4. Identification and quantification of fatty acids by GC–FID and –MS

GC analysis was performed using a Trace 1310 GC (ThermoFisher scientific, Waltham, MA, USA) equipped with an RT2560 capillary column (100 m × 0.25 mm, 0.2 µm film thickness) (Restek, Lisses, France), an automatic injector and a flame ionisation detector (FID) set at 255°C. Samples were injected with hydrogen as carrier gas at a constant pressure of 200 kPa into the column programmed for ramped oven temperatures as described previously (Ngo Njembe et al., 2021a). FAME were identified by comparing their retention times to authentic standards of known composition (Larodan, Solna, Sweden). Peak areas and fatty acid concentrations were calculated using the ChromQuest 5.0 software (ThermoFisher Scientific).

The lipid extracts were also analysed by GC-MS to characterize fatty acid peaks that could not be identified with GC-FID and to confirm the identification of the fatty acids detected. For GC-MS analysis, a Trace 1310 GC was equipped with an ISQ QD mass spectrometer (MS) detector (ThermoFisher Scientific, Waltham, MA, USA). The column and the oven temperature program were identical to the GC-FID analysis. The column operating conditions of the GC-MS analysis were as follows: helium carrier gas at 2 ml/min; a split/splitless inlet at 250°C; a split ratio of 10:1; a transfer line temperature of 250°C. The parameters used for mass spectrometry analyses were as follows: ionization energy at 70 eV; ion source temperature of 300°C; dwell time of 0.2 s; a mass range of 40 - 400 *m/z*. The system operated and the data was processed using the Thermo Scientific Chromeleon 7 Chromatography Data System (CDS) software. Fatty acid values

were expressed as milligrams per gram of material (mg/g) or as a percentage (%) of the total fatty acids identified.

6.2.5. Statistical analysis

Egg weight, yolk weight and fatty acid composition of egg yolks were analysed by two-way analysis of variance (ANOVA) using JMP 15 (SAS institute inc., Cary, NC, USA), to determine the effects of dietary treatments, the duration of the experiment and their interaction. Treatments (F-OL and FS-PSO) and periods (1 to 6) were the fixed effects and least squares means, adjusted using the Tukey-Kramer test, were compared for significant differences set at $p < 0.05$. The difference between dietary treatments in terms of fatty acids in tissues was determined using a two-tailed t-test and the results were expressed as means and standard errors of the mean.

6.3. Results

6.3.1. Dietary fats and hens' performance

CLnA were only present in the PSO-supplemented diet (Table 6.1). The predominantly abundant CLnA was PunA, accounting for approximately 73% of the total fatty acids in PSO. Its concentration was 39.53 mg/g in the FS-PSO diet. Five other CLnA present at low levels in PSO, α-eleostearic acid (*C18:3c9,t11,t13*), α-calendic acid (*C18:3t8,t10,c12*), catalpic acid (*C18:3t9,t11,c13*), β-eleostearic acid (*C18:3t9,t11,t13*), and β-calendic acid (*C18:3t8,t10,t12*), were detected in the hens' diet. Inclusion of flaxseeds resulted in the incorporation of a high level of ALA in the FS-PSO diet. The F-OL diet had a very high content in OA (91.06 mg/g). Other fatty acids such as palmitic acid (*C16:0*), stearic acid (*C18:0*), and linoleic acid (*C18:2c9,c12*) were also higher in the F-OL diet.

Egg production was similar in hens from the two dietary treatments, averaging about 85%, over the entire experimental period (Table 6.2). Similarly, egg weight and yolk weight were not affected by the dietary treatments or the duration of feeding. Total fat was also similar in yolks from the two dietary treatments (as mean ± SEM: F-OL = 31.08 ± 0.59%; FS-PSO = 32.45 ± 0.44 %).

6.3.2. Fatty acid composition of egg yolks

The fatty acid compositions of the yolks (in mg/g of yolk) as a function of the two dietary treatments and over the entire feeding duration are presented in Table 6.3. Total saturated fatty acids (SFA) showed a highly significant ($p < 0.0001$) time effect for both F-OL and FS-PSO diets. Palmitic and stearic acids decreased in the yolks in both groups all along the experiment, with the lowest levels seen at period 6. However, higher concentrations of these fatty acids were observed with the FS-PSO diet compared to F-OL.

As compared with the FS-PSO group, feeding the F-OL diet to hens resulted in higher concentrations of hypogeic acid (*C16:1c7*), OA and cis-vaccenic acid (*C18:1c11*) in yolk lipids. Unlike other monounsaturated fatty acids (MUFA), there was no significant effect of the diet on palmitoleic acid (*C16:1c9*), whose levels within periods were not significantly different between treatment groups throughout the experiment. The duration of the experiment resulted in a further decrease in MUFA of FS-PSO egg yolks. The same observation was made in F-OL yolks for palmitoleic and cis-vaccenic acids. In contrast, there were no significant differences in OA levels in F-OL eggs throughout the feeding duration.

Table 6.1. Main fatty acid composition of fat sources and experimental diets

Fatty acids	***Fat sources***			***Dietary treatments***	
	Olive oil	Flaxseed oil	Pomegranate seed oil	F-OL	FS-PSO
	% wt. of total fatty acids identified			mg/g of feed	
Palmitic acid (C16:0)	9.68	5.27	2.61	19.36	9.08
Stearic acid (C18:0)	3.18	4.20	1.74	4.74	2.96
Oleic acid (C18:1c9)	78.46	16.66	4.39	91.06	14.74
Cis-vaccenic acid (C18:1c11)	1.61	0.67	0.40	2.16	0.67
Linoleic acid (C18:2c9,c12)	4.82	15.22	5.67	23.63	21.16
Alpha-linolenic acid (C18:3c9,c12,c15)	0.71	56.80	0.73	2.09	16.57
Punicic acid (C18:3c9,t11,c13)	ND	ND	73.21	ND	39.53
Alpha-eleostearic acid (C18:3c9,t11,t13)*	ND	ND	0.64	ND	0.35
Alpha-calendic acid (C18:3t8,t10,c12)*	ND	ND	0.82	ND	0.44
Catalpic acid (C18:3t9,t11,c13)*	ND	ND	4.85	ND	2.62
Beta-eleostearic acid (C18:3t9,t11,t13)*	ND	ND	1.99	ND	1.08
Beta-calendic acid (C18:3t8,t10,t12)*	ND	ND	0.56	ND	0.30
Σ SFA	13.56	9.99	5.70	25.74	13.53
Σ MUFA	80.76	17.44	5.51	94.26	15.52
Σ n-6 PUFA	4.82	15.27	5.70	23.63	21.26
Σ n-3 PUFA	0.84	57.26	1.02	2.25	16.69
Σ CLnA	ND	ND	82.07	ND	44.36

*Calculated as punicic acid equivalents. F-OL: hens' feed supplemented with 10% (wt.) olive oil; FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil; ND: not detected; Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ n-6 PUFA: total omega-6 polyunsaturated fatty acids; Σ n-3 PUFA: total omega-3 polyunsaturated fatty acids; Σ CLnA: total conjugated linolenic acids.

Table 6.2. Effect of dietary treatments on hens' performance, egg weight and egg yolk weight

	Diet effect			**Diet × Period effect**						***p*-Value**		
	Dietary treatments		SE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Diet	Period	Diet × Period
Laying rate (%)	F-OL	85.75	1.61	76.97	86.81	89.65	89.85	85.69	85.51	0.75	0.12	0.93
	FS-PSO	85.01		79.19	82.89	92.17	85.37	85.44	84.98			
Egg weight (g)	F-OL	62.86	0.47	63.41	65.54	60.92	60.97	62.63	63.64	0.06	0.06	0.11
	FS-PSO	61.60		60.11	61.04	62.34	60.14	62.44	63.53			
Yolk weight (g)	F-OL	16.57	0.13	16.71	17.28	16.06	16.07	16.51	16.78	0.07	0.05	0.12
	FS-PSO	16.24		15.84	16.09	16.43	15.85	16.46	16.75			

Each period is 28 days and represents the average value of 4 weeks (six eggs per treatment were analysed separately each week).
Data are presented as least square mean (LSM) ± standard error (SE) between treatments (diet effect) or as LSM between periods (diet × period effect).
F-OL: hens' feed supplemented with 10% (wt.) olive oil. FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil.

There was an effect of treatment on the amount of total n-6 PUFA in egg yolks, although there was no interaction between dietary treatment and duration of feeding. However, the treatment effect varied depending on the n-6 fatty acids. The accumulation of linoleic acid (*C18:2c9,c12*) was reduced in both egg types. The decrease was greater in F-OL eggs while the diet contained a higher amount of this fatty acid (Table 6.1). The accumulations of arachidonic acid (*C20:4c5,c8,c11,c14*) and n-6 docosapentaenoic acid (n-6 DPA; *C22:5c4,c7,c10,c13,c16*) were lower in FS-PSO eggs, whereas their concentrations remained relatively constant in F-OL eggs throughout the experiment.

The concentration of ALA increased in yolks of hens fed on flaxseeds and this increase persisted until the end of the experiment. In contrast, ALA in the yolks produced by hens fed the F-OL diet remained at a relatively low concentration throughout the experiment and tended to decrease. Including olive oil in the diet did not affect DHA concentration in yolks of the F-OL group, and the accumulation of synthesized DHA from ALA was 2.2-fold higher in the yolk of FS-PSO eggs compared to F-OL. The effects of dietary treatments on n-3 DPA (*C22:5c7,c10,c13,c16,c19*) concentrations in yolk lipids were similar to those seen with DHA, namely an increase in FS-PSO eggs and no significant change in F-OL eggs. A significant ($p < 0.0001$) diet effect was observed in the deposition of eicosapentaenoic acid (EPA, *C20:5c5,c8,c11,c14,c17*), although this fatty acid was absent in F-OL eggs and deposited in very low concentrations in FS-PSO yolk lipids. There was no time effect on the total concentration of n-3 PUFA in egg yolks.

Levels of CLnA in egg lipids were greatly ($p < 0.0001$) influenced by the incorporation of PSO in the diet, with PunA, found only in eggs from the FS-PSO group. CLnA other than PunA were also deposited in yolk lipids. Expressed as PunA equivalents, the concentrations of these CLnA, namely α-eleostearic acid (*C18:3c9,t11,t13*), α-calendic acid (*C18:3t8,t10,c12*), catalpic acid (*C18:3t9,t11,c13*), β-eleostearic acid (*C18:3t9,t11,t13*), and β-

calendic acid (*C18:3t8,t10,t12*) clearly reflected their levels in the FS-PSO diet. Another CLnA, octadeca-6,9,11-trienoic acid (c6,c9,t11-CLnA (*C18:3c6,c9,t11*)), not identified in the FS-PSO diet, was detected in egg yolk lipids. Its level significantly increased in the FS-PSO group as compared to trace amounts in the F-OL group. Regarding the CLA isomers, the concentration of RmA in the egg yolks of the FS-PSO group was drastically higher than in the F-OL group. Furthermore, it increased as the experiment progressed. The maximum concentration (40.19 mg/g of yolk) was seen at period 6. Some other conjugated dienoic acids, including cis-7, trans-9-hexadecadienoic acid (*C16:2c7,t9*) and trans-9, trans-11-octadecadienoic acid (t9,t11-CLA (*C18:2t9,t11*)) were detected in egg yolks. While remaining considerably lower compared to RmA, their concentrations were largely higher in FS-PSO eggs than in F-OL eggs where they remained in trace amounts.

6.3.3. Distribution of OA, ALA, RmA and PunA of yolk into lipid classes

Figure 6.1 shows the distribution of OA, ALA and DHA, as well as that of RmA and PunA in the case of FS-PSO eggs, into the different lipid classes (TG, PL and FFA), and Table 6.4 presents the fatty acid composition of TG and PL in percentages by weight of total fatty acids. Fatty acids in yolk were deposited primarily in TG and PL, which accounted for 60 to 65% and 29 to 33% of yolk lipids, respectively. The distribution of OA was the same in lipid classes (TG, 77%; PL, 20%; FFA, 3%) for both types of eggs. However, OA proportions in TG and PL in F-OL eggs were two times higher than in FS-PSO eggs. The enrichment of ALA upon feeding flaxseeds was mainly in yolk TG, 88% of ALA being deposited in the TG fraction, resulting in an average proportion of 2.89% ALA in the total fatty acids in TG. Only 10% of ALA was accumulated in the PL fraction of FS-PSO yolks. The low ALA content in F-OL eggs exhibited almost the same distribution pattern (TG, 82% and PL, 15%). In contrast, 87% of the DHA accumulated was found in the PL fraction

regardless of its concentration in the yolk. Supplementation of the hens' diet with PSO resulted in the accumulation of PunA and RmA mainly in the TG fraction of yolk lipids. Besides, these two fatty acids were distributed in similar pattern between the different lipid classes. Indeed, 73% of PunA and 72% of RmA were accumulated in the TG fraction, whereas only 25% of PunA and 26% of RmA were found in the PL fraction.

Table 6.3. Effect of supplementing the hen's diet with olive oil or flaxseeds and pomegranate seed oil on the fatty acid composition of the yolks

		Diet effect		**Diet × Period effect**						***p*-Value**		
Fatty acids (mg/g of yolk)	Dietary treatments		SE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Diet	Period	Diet × Period
Palmitic acid (C16:0)	F-OL	59.15^{a}	0.77	75.85^{a}	60.04^{bcd}	53.50^{d}	56.98^{d}	56.57^{d}	51.93^{d}	0.0031	<0.0001	0.0858
	FS-PSO	62.34^{b}		74.49^{a}	65.56^{b}	59.44^{bcd}	64.34^{bc}	57.34^{cd}	52.88^{d}			
Hypogeic acid (C16:1c7)	F-OL	4.98^{a}	0.09	5.14^{ab}	4.57^{b}	4.72^{ab}	5.33^{ab}	5.55^{a}	4.58^{b}	<0.0001	0.0045	0.0186
	FS-PSO	2.38^{b}		3.04^{c}	2.10^{c}	2.32^{c}	2.27^{c}	2.24^{c}	2.32^{c}			
Palmitoleic acid (C16:1c9)	F-OL	3.72^{a}	0.11	6.90^{a}	3.54^{b}	2.94^{bc}	3.09^{bc}	3.24^{bc}	2.57^{bc}	0.0667	<0.0001	0.1002
	FS-PSO	3.43^{a}		5.96^{ab}	3.73^{b}	2.82^{bc}	3.40^{b}	2.57^{bc}	2.08^{c}			
Cis-7, trans-9-hexadecadienoic acid (C16:2c7,t9)	F-OL	0.07^{a}	0.07	0.12^{c}	0.06^{c}	0.06^{c}	0.07^{c}	0.06^{c}	Traces^{c}	<0.0001	0.0173	0.0059
	FS-PSO	3.75^{b}		2.82^{b}	3.64^{ab}	3.88^{a}	3.77^{a}	4.08^{a}	4.31^{a}			
Stearic acid (C18:0)	F-OL	20.27^{a}	0.31	24.32^{a}	19.89^{bc}	18.84^{c}	20.36^{bc}	19.62^{c}	18.62^{c}	<0.0001	<0.0001	0.2497
	FS-PSO	23.49^{b}		25.26^{a}	24.63^{a}	22.49^{abc}	24.22^{a}	23.01^{ab}	21.32^{abc}			
Oleic acid (C18:1c9)	F-OL	153.40^{a}	1.70	154.86^{a}	160.25^{a}	144.25^{a}	158.13^{a}	156.14^{a}	146.78^{a}	<0.0001	<0.0001	<0.0001
	FS-PSO	72.59^{b}		103.87^{b}	70.04^{c}	67.19^{c}	71.52^{c}	63.30^{c}	59.68^{c}			
Cis-vaccenic acid (C18:1c11)	F-OL	4.30^{a}	0.05	5.78^{a}	4.25^{b}	4.00^{bc}	4.16^{bc}	4.03^{bc}	3.56^{c}	<0.0001	<0.0001	0.3349
	FS-PSO	2.45^{b}		4.23^{bc}	2.34^{d}	2.28^{d}	2.29^{d}	1.86^{d}	1.68^{d}			
Linoleic acid (C18:2c9,c12)	F-OL	33.11^{a}	0.63	45.48^{ab}	30.17^{de}	29.70^{de}	32.67^{cde}	31.75^{de}	28.86^{e}	<0.0001	<0.0001	0.4329
	FS-PSO	39.46^{b}		49.45^{a}	36.65^{cd}	39.63^{bc}	37.98^{c}	36.15^{cde}	36.89^{cde}			

Table 6.3. Effect of supplementing the hen's diet with olive oil or flaxseeds and pomegranate seed oil on the fatty acid composition of the yolks (continued)

		Diet effect		**Diet × Period effect**						***p*-Value**		
Fatty acids (mg/g of yolk)	Dietary treatments		SE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Diet	Period	Diet × Period
Rumenic acid (C18:2c9,t11)	F-OL	0.13^{a}	0.47	0.28^{c}	0.16^{c}	0.08^{c}	0.08^{c}	0.15^{c}	0.06^{c}	<0.0001	0.0026	0.0016
	FS-PSO	35.93^{b}		28.64^{b}	35.58^{a}	35.25^{ab}	37.70^{a}	38.23^{a}	40.19^{a}			
Trans-9, trans-11-octadeca-dienoic acid (C18:2t9,t11)*	F-OL	ND^{a}	0.02	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	<0.0001	0.6133	0.4942
	FS-PSO	0.96^{b}		1.05^{a}	1.01^{a}	0.92^{a}	0.92^{a}	0.92^{a}	0.92^{a}			
Cis-6, cis-9, trans-11-octadecatrienoic acid (C18:3c6,c9,t11)	F-OL	0.05^{a}	0.02	0.07^{b}	0.06^{b}	ND^{b}	0.07^{b}	0.06^{b}	0.05^{b}	<0.0001	0.7764	0.7149
	FS-PSO	0.88^{b}		0.94^{a}	0.89^{a}	0.94^{a}	0.82^{a}	0.84^{a}	0.84^{a}			
Alpha-linolenic acid (C18:3c9,c12,c15)	F-OL	0.91^{a}	0.19	1.50^{c}	0.86^{c}	0.74^{c}	0.84^{c}	0.85^{c}	0.67^{c}	<0.0001	0.4431	0.0327
	FS-PSO	6.15^{b}		4.76^{b}	6.85^{ab}	5.61^{ab}	6.03^{ab}	6.35^{ab}	7.29^{a}			
Punicic acid (C18:3c9,t11,c13)	F-OL	ND^{a}	0.40	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	<0.0001	0.0007	0.0007
	FS-PSO	20.00^{b}		21.47^{ab}	19.14^{b}	16.93^{b}	18.05^{b}	24.16^{a}	20.23^{ab}			
Alpha-eleostearic acid (C18:3c9,t11,t13)**	F-OL	ND^{a}	0.004	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	<0.0001	0.0023	0.0023
	FS-PSO	0.25^{b}		0.30^{a}	0.26^{ab}	0.26^{ab}	0.21^{c}	0.25^{abc}	0.23^{bc}			
Alpha-calendic acid (C18:3t8,t10,c12)**	F-OL	ND^{a}	0.004	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	<0.0001	<0.0001	<0.0001
	FS-PSO	0.20^{b}		0.25^{ab}	0.26^{a}	0.20^{b}	0.23^{ab}	0.14^{c}	0.12^{c}			

Table 6.3. Effect of supplementing the hen's diet with olive oil or flaxseeds and pomegranate seed oil on the fatty acid composition of the yolks (continued)

		Diet effect		**Diet × Period effect**						***p*-Value**		
Fatty acids (mg/g of yolk)	Dietary treatments		SE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Diet	Period	Diet × Period
Catalpic acid	F-OL	ND^{a}	0.02	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	<0.0001	0.1392	0.1426
(C18:3t9,t11,c13)**	FS-PSO	1.06^{b}		1.18^{ab}	1.05^{ab}	1.16^{a}	0.91^{b}	1.07^{ab}	1.02^{ab}			
Beta-eleostearic acid	F-OL	ND^{a}	0.01	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	<0.0001	0.0597	0.0600
(C18:3t9,t11,t13)**	FS-PSO	0.69^{b}		0.75^{ab}	0.68^{ab}	0.69^{ab}	0.58^{b}	0.74^{a}	0.70^{ab}			
Beta-calendic acid	F-OL	ND^{a}	0.01	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	<0.0001	0.1362	0.1362
(C18:3t8,t10,t12)**	FS-PSO	0.33^{b}		0.38^{a}	0.34^{ab}	0.34^{ab}	0.29^{b}	0.34^{ab}	0.32^{ab}			
Arachidonic acid	F-OL	5.66^{a}	0.08	5.91^{ab}	5.42^{ab}	5.34^{abc}	5.86^{ab}	6.01^{a}	5.40^{abc}	<0.0001	<0.0001	<0.0001
(C20:4c5,c8,c11,c14)	FS-PSO	3.95^{b}		5.05^{bc}	3.78^{de}	4.45^{cd}	3.82^{de}	3.61^{de}	3.00^{e}			
Eicosapentaenoic acid	F-OL	ND^{a}	0.005	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	<0.0001	0.1236	0.1289
(C20:5c5,c8,c11,c14,c17)	FS-PSO	0.16^{b}		0.19^{a}	0.18^{ab}	0.12^{b}	0.16^{ab}	0.16^{ab}	0.15^{ab}			
n-6 Docosapentaenoic acid	F-OL	1.36^{a}	0.03	1.47^{ab}	1.18^{b}	1.52^{ab}	1.51^{a}	1.33^{ab}	1.16^{bc}	<0.0001	<0.0001	0.0012
(C22:5c4,c7,c10,c13,c16)	FS-PSO	0.35^{b}		0.80^{c}	0.31^{d}	0.34^{d}	0.29^{d}	0.20^{d}	0.16^{d}			
n-3 Docosapentaenoic acid	F-OL	0.48^{a}	0.40	0.44^{d}	0.41^{d}	0.49^{cd}	0.56^{cd}	0.53^{cd}	0.41^{d}	<0.0001	<0.0001	0.0002
(C22:5c7,c10,c13,c16,c19)	FS-PSO	1.39^{b}		0.92^{bc}	1.72^{a}	1.25^{ab}	1.44^{a}	1.66^{a}	1.35^{ab}			
Docosahexaenoic acid	F-OL	2.22^{a}	0.10	2.57^{c}	2.22^{c}	2.27^{c}	2.28^{c}	2.08^{c}	1.95^{c}	<0.0001	0.0898	0.0020
(C22:6c4,c7c10,c13,c16,c19)	FS-PSO	4.90^{b}		3.82^{b}	5.06^{a}	5.54^{a}	5.14^{a}	4.80^{ab}	5.03^{ab}			

Table 6.3. Effect of supplementing the hen's diet with olive oil or flaxseeds and pomegranate seed oil on the fatty acid composition of the yolks (continued)

		Diet effect		**Diet × Period effect**						***p*-Value**		
Fatty acids (mg/g of yolk)	Dietary treatments		SE	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Diet	Period	Diet × Period
Σ SFA	F-OL	81.79^{a}	1.02	103.21^{a}	82.50^{de}	74.47^{e}	79.72^{e}	78.32^{e}	72.51^{e}	<0.0001	<0.0001	0.0934
	FS-PSO	88.87^{b}		102.89^{ab}	93.48^{abc}	84.98^{cde}	91.75^{bcd}	83.23^{cde}	76.86^{e}			
Σ MUFA	F-OL	166.99^{a}	1.87	171.46^{a}	173.58^{a}	156.68^{a}	171.65^{a}	170.07^{a}	158.49^{a}	<0.0001	<0.0001	0.0001
	FS-PSO	80.95^{b}		116.79^{b}	79.01^{c}	69.58^{c}	80.40^{c}	70.81^{c}	69.12^{c}			
Σ n-6 PUFA	F-OL	42.03^{a}	0.71	56.63^{a}	38.18^{bc}	38.18^{bc}	41.78^{bc}	40.67^{bc}	36.76^{c}	0.0015	<0.0001	0.2487
	FS-PSO	45.28^{b}		56.43^{a}	42.37^{bc}	46.23^{b}	43.80^{bc}	41.45^{bc}	41.38^{bc}			
Σ n-3 PUFA	F-OL	3.67^{a}	0.30	4.53^{a}	3.55^{a}	3.57^{a}	3.74^{a}	3.54^{a}	3.08^{a}	<0.0001	0.6020	0.5341
	FS-PSO	14.14^{b}		14.55^{b}	14.89^{b}	12.73^{b}	13.87^{b}	14.00^{b}	14.81^{b}			
Σ CLA	F-OL	0.20^{a}	0.46	0.38^{c}	0.22^{c}	0.13^{c}	0.15^{c}	0.21^{c}	0.13^{c}	<0.0001	0.0016	0.0007
	FS-PSO	37.04^{b}		29.87^{b}	36.79^{a}	36.20^{a}	38.81^{a}	40.40^{a}	41.29^{a}			
Σ CLnA	F-OL	0.05^{a}	0.39	0.07^{c}	0.06^{c}	ND^{c}	0.07^{c}	0.06^{c}	0.05^{c}	<0.0001	0.0001	0.0002
	FS-PSO	23.34^{b}		25.28^{ab}	22.62^{b}	20.30^{b}	20.85^{b}	27.53^{a}	23.47^{ab}			

Each period is 28 days and represents the average value of 4 weeks (six eggs per treatment were analysed separately each week). Data are presented as least square mean (LSM) ± standard error (SE) between treatments (diet effect) or as LSM between periods (diet × period effect). Values with no common superscript letters (a, b, c, d, e) between treatments (diet effect) or between periods (diet x period) are significantly different at $p < 0.05$, using 2-way ANOVA followed by Tukey's test. "Traces" means detectable but too low to be quantified (<0.05 mg/g yolk). F-OL: hens' feed supplemented with 10% (wt.) olive oil. FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil. *Calculated as rumenic acid equivalents. **Calculated as punicic acid equivalents. ND: not detected; Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ n-6 PUFA: total omega-6 polyunsaturated fatty acids; Σ n-3 PUFA: total omega-3 polyunsaturated fatty acids; Σ CLA: total conjugated linoleic acids; Σ CLnA: total conjugated linolenic acids.

Figure 6.1. Incorporation of oleic acid (OA), α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) into different lipid fractions (triglycerides (TG), phospholipids (PL), and free fatty acids (FFA)) of egg yolk. F-OL: hens' feed supplemented with 10% (wt.) olive oil. FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil.

Table 6.4. Effect of dietary fats on fatty acid compositions of triglycerides and phospholipids of yolks

	Triglycerides		**Phospholipids**	
% wt. of total fatty acids identified	F-OL	FS-PS	F-OL	FS-PS
Palmitic acid (C16:0)	17.91 ± 1.93[a]	19.92 ± 1.32[b]	25.07 ± 0.35[a]	23.40 ± 0.49[b]
Hypogeic acid (C16:1c7)	2.38 ± 0.12[a]	0.96 ± 0.09[b]	0.56 ± 0.03[a]	0.17 ± 0.01[b]
Palmitoleic acid (C16:1c9)	1.12 ± 0.08[a]	1.09 ± 0.07[a]	0.41 ± 0.02[a]	0.27 ± 0.01[b]
Cis-7, trans-9-hexadecadienoic acid (C16:2cis7,t9)	0.01 ± 0.004[a]	1.87 ± 0.27[b]	0.02 ± 0.002[a]	0.20 ± 0.01[a]
Stearic acid (C18:0)	4.22 ± 0.20[a]	5.60 ± 0.21[b]	17.83 ± 0.50[a]	19.39 ± 0.45[b]
Oleic acid (C18:1c9)	30.45 ± 9.19[a]	17.90 ± 3.84[b]	28.34 ± 0.55[a]	12.58 ± 0.36[b]
Cis-vaccenic acid (C18:1c11)	30.08 ± 8.64[a]	8.85 ± 3.47[b]	1.12 ± 0.04[a]	0.38 ± 0.01[b]
Linoleic acid (C18:2c9,c12)	11.02 ± 0.34[a]	13.62 ± 0.87[a]	12.39 ± 0.31[a]	12.04 ± 0.26[a]
Rumenic acid (C18:2c9,t11)	0.06 ± 0.01[a]	14.62 ± 0.41[b]	0.04 ± 0.01[a]	9.64 ± 0.32[b]
Trans-9, trans-11-octadeca-dienoic acid (C18:2t9,t11)*	ND[a]	0.01 ± 0.005[b]	ND[a]	ND[a]
Cis-6, cis-9, trans-11-octadecatrienoic acid (C18:3c6,c9,t11)	0.01 ± 0.004[a]	0.24 ± 0.010[b]	0.01 ± 0.002[a]	0.56 ± 0.03[b]
Alpha-linolenic acid (C18:3c9,c12,c15)	0.30 ± 0.01[a]	2.89 ± 0.24[b]	0.11 ± 0.004[a]	0.60 ± 0.04[b]
Punicic acid (C18:3c9,t11,c13)	ND[a]	7.90 ± 1.15[b]	ND[a]	4.98 ± 0.3[b]
Alpha-eleostearic acid (C18:3c9,t11,t13)**	ND[a]	0.08 ± 0.004[b]	ND[a]	0.05 ± 0.002[b]
Alpha-calendic acid (C18:3t8,t10,c12)**	ND[a]	0.06 ± 0.01[b]	ND[a]	0.05 ± 0.004[b]
Catalpic acid (C18:3t9,t11,c13)**	ND[a]	0.35 ± 0.07[b]	ND[a]	0.28 ± 0.01[b]
Beta-eleostearic acid (C18:3t9,t11,t13)**	ND[a]	0.24 ± 0.02[b]	ND[a]	0.19 ± 0.01[b]

Table 6.4. Effect of dietary fats on fatty acid compositions of triglycerides and phospholipids of yolks (continued)

	Triglycerides		**Phospholipids**	
% wt. of total fatty acids identified	F-OL	FS-PS	F-OL	FS-PS
Beta-calendic acid (C18:3t8,t10,t12)**	ND[a]	0.11 ± 0.01[b]	ND[a]	0.07 ± 0.004[b]
Arachidonic acid (C20:4c5,c8,c11,c14)	0.72 ± 0.10[a]	0.41 ± 0.06[b]	6.48 ± 0.28[a]	4.51 ± 0.14[b]
Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)	ND[a]	0.05 ± 0.01[b]	ND[a]	0.16 ± 0.01[b]
n-6 Docosapentaenoic acid (C22:5c4,c7,c10,c13,c16)	0.13 ± 0.03[a]	0.03 ± 0.01[b]	1.40 ± 0.19[a]	0.26 ± 0.03[b]
n-3 Docosapentaenoic acid (C22:5c7,c10,c13,c16,c19)	0.12 ± 0.008[a]	0.39 ± 0.03[b]	0.43 ± 0.01[a]	1.41 ± 0.10[b]
Docosahexaenoic acid (C22:6c4,c7c10,c13,c16,c19)	0.20 ± 0.03[a]	0.48 ± 0.07[b]	2.83 ± 0.13[a]	6.55 ± 0.38[b]
Σ SFA	22.48 ± 0.63[a]	26.20 ± 0.49[b]	44.00 ± 0.86[a]	43.35 ± 0.92[a]
Σ MUFA	64.44 ± 0.81[a]	29.01 ± 0.73[b]	30.90 ± 0.57[a]	13.60 ± 0.32[b]
Σ n-6 PUFA	12.25 ± 0.43[a]	14.52 ± 0.27[b]	21.44 ± 0.38[a]	17.71 ± 0.35[b]
Σ n-3 PUFA	0.64 ± 0.03[a]	3.91 ± 0.23[b]	3.41 ± 0.12[a]	8.83 ± 0.43[b]
Σ CLA	0.07 ± 0.01[a]	14.67 ± 0.43[b]	0.05 ± 0.01[a]	9.66 ± 0.33[b]
Σ CLnA	0.01 ± 0.004[a]	8.98 ± 0.33[b]	0.01 ± 0.002[a]	6.18 ± 0.28[b]

Data are means ± standard errors of the mean (n = 24).

Values with no common superscript letters (a, b) are significantly different at $p < 0.05$, using two-sample t-test.

F-OL: hens' feed supplemented with 10% (wt.) olive oil. FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil. *Calculated as rumenic acid equivalents. **Calculated as punicic acid equivalents. ND: not detected; Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ n-6 PUFA: total omega-6 polyunsaturated fatty acids; Σ n-3 PUFA: total omega-3 polyunsaturated fatty acids; Σ CLA: total conjugated linoleic acids; Σ CLnA: total conjugated linolenic acids.

6.3.4. Incorporation of dietary lipids on tissues of hens

Analysis of the liver, heart, muscle and adipose tissue of the hens showed that the composition of fatty acids in these tissues was greatly affected by dietary lipids (Table 6.5). In general, palmitic acid, OA and linoleic acid were the major components of both TG and PL fractions. Stearic acid and long chain PUFA were present in significant amounts in PL, but only very small amounts of these fatty acids were detectable in TG. Unlike ALA, which accumulated almost exclusively in TG, RmA and PunA were deposited in appreciable proportions in both TG and PL fractions.

As observed with eggs, the CLnA present in the feed were incorporated into the hens' tissues from the FS-PSO group in proportions corresponding to those provided by the diet. Apart from PunA, the other food-borne CLnA were at trace levels. PunA, together with c6,c9,t11-CLnA, were the isomers found in substantial amounts in hens' tissues. In addition, the proportions of c6,c9,t11-CLnA were higher than that of PunA in the PL fraction in tissues. CLA were also present in all the tissues examined. RmA was the predominant CLA isomer and it was found in tissue fat at higher levels than PunA. In contrast, no CLnA was detected in the tissues of animals fed with the F-OL diet, whereas CLA were detected at very low levels.

There was a substantial increase in OA in all tissues in the group receiving the F-OL diet. Palmitoleic acid was similar between both dietary treatments, while hypogeic and cis-vaccenic acids were higher in hens fed the F-OL diet. Among SFA, there was no difference in the amounts of palmitic and stearic acids in muscle. In contrast, the proportions of these two fatty acids were significantly increased in the heart and adipose tissue of birds fed with the FS-PSO diet. In the liver, the contents of palmitic and stearic acids were also increased in the FS-PSO group, although the increase in palmitic acid was not significant. ALA was higher in the fat of hens fed flaxseeds, resulting in a significant increase in the total n-3

PUFA proportion in the TG fraction. In parallel, a significant increase in the long-chain n-3 PUFA, DHA and n-3 DPA, was seen in the PL fraction, when compared with the F-OL group. EPA was found at very low levels in both groups. Linoleic acid significantly accumulated in the fat of all tissues when feeding the FS-PSO diet. In contrast, the proportions of the long-chain n-6 PUFA, arachidonic acid and n-6 DPA, were significantly reduced in the PL fraction compared to the F-OL group.

Table 6.5. Fatty acid composition of triglycerides and phospholipids in the liver, heart, adipose tissue and muscle of hens fed with the experimental diets

		Liver		**Heart**		**Muscle**		**Adipose tissue**	
% total fatty acids	Lipid fractions	F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO
Palmitic acid	TG	16.55 ± 0.77^{a}	18.15 ± 1.02^{a}	12.23 ± 0.34^{a}	14.25 ± 0.34^{b}	13.99 ± 0.41^{a}	15.59 ± 0.49^{a}	11.04 ± 0.29^{a}	13.62 ± 0.29^{b}
	PL	22.14 ± 0.15^{a}	20.90 ± 0.51^{b}	20.48 ± 0.36^{a}	19.49 ± 0.46^{a}	17.88 ± 0.43^{a}	18.09 ± 0.80^{a}	ND	ND
Hypogeic acid	TG	1.53 ± 0.10^{a}	0.70 ± 0.08^{b}	0.66 ± 0.04^{a}	0.47 ± 0.05^{b}	0.94 ± 0.06^{a}	0.65 ± 0.04^{b}	0.52 ± 0.03^{a}	0.39 ± 0.03^{b}
	PL	0.41 ± 0.03^{a}	0.16 ± 0.02^{b}	0.14 ± 0.005^{a}	0.07 ± 0.01^{b}	0.14 ± 0.01^{a}	0.05 ± 0.01^{b}	ND	ND
Palmitoleic acid	TG	0.91 ± 0.16^{a}	0.83 ± 0.04^{a}	0.85 ± 0.10^{a}	0.98 ± 0.11^{a}	0.89 ± 0.11^{a}	1.34 ± 0.22^{a}	0.64 ± 0.06^{a}	0.80 ± 0.06^{a}
	PL	0.37 ± 0.06^{a}	0.28 ± 0.02^{a}	0.10 ± 0.01^{a}	0.08 ± 0.01^{a}	0.12 ± 0.004^{a}	0.13 ± 0.01^{a}	ND	ND
Stearic acid	TG	5.19 ± 0.30^{a}	7.22 ± 0.50^{b}	3.21 ± 0.11^{a}	4.20 ± 0.25^{b}	4.27 ± 0.13^{a}	5.92 ± 0.92^{a}	3.30 ± 0.13^{a}	4.49 ± 0.42^{b}
	PL	18.54 ± 0.55^{a}	17.13 ± 0.88^{a}	20.76 ± 0.61^{a}	20.74 ± 0.75^{a}	18.65 ± 0.49^{a}	16.89 ± 1.24^{a}	ND	ND
Oleic acid	TG	60.22 ± 0.65^{a}	27.91 ± 1.88^{b}	48.28 ± 0.30^{a}	33.23 ± 1.18^{b}	58.49 ± 0.45^{a}	35.14 ± 1.66^{b}	63.42 ± 0.24^{a}	30.88 ± 2.01^{b}
	PL	26.16 ± 0.68^{a}	12.12 ± 0.85^{b}	13.65 ± 0.49^{a}	5.19 ± 0.57^{b}	21.10 ± 0.35^{a}	5.84 ± 0.58^{b}	ND	ND
Cis-vaccenic acid	TG	1.37 ± 0.07^{a}	1.03 ± 0.08^{b}	14.76 ± 0.09^{a}	1.23 ± 0.05^{b}	1.84 ± 0.02^{a}	1.52 ± 0.11^{b}	2.47 ± 0.11^{a}	1.69 ± 0.12^{b}
	PL	1.04 ± 0.03^{a}	0.44 ± 0.04^{b}	2.00 ± 0.16^{a}	0.69 ± 0.10^{b}	2.07 ± 0.05^{a}	0.82 ± 0.08^{b}	ND	ND
Linoleic acid	TG	11.59 ± 1.23^{a}	17.60 ± 0.37^{b}	17.77 ± 0.55^{a}	23.85 ± 0.67^{b}	17.05 ± 0.68^{a}	20.05 ± 0.78^{b}	16.40 ± 0.33^{a}	23.63 ± 0.60^{b}
	PL	11.66 ± 0.36^{a}	11.51 ± 0.45^{a}	10.61 ± 0.54^{a}	13.71 ± 0.53^{b}	16.02± 0.75^{a}	24.15 ± 0.81^{b}	ND	ND
Rumenic acid	TG	0.02 ± 0.004^{a}	9.88 ± 1.09^{b}	0.01 ± 0.001^{a}	9.04 ± 0.85^{b}	0.02 ± 0.003^{a}	8.68 ± 1.05^{b}	0.01 ± 0.005^{a}	10.07 ± 1.33^{b}
	PL	0.02 ± 0.001^{a}	8.34 ± 1.10^{b}	Traces^{a}	5.73 ± 0.47^{b}	0.02 ± 0.01^{a}	5.26 ± 0.48^{b}	ND	ND

Table 6.5. Fatty acid composition of triglycerides and phospholipids in the liver, heart, adipose tissue and muscle of hens fed with the experimental diets (continued)

% total fatty acids	Lipid fractions	Liver		Heart		Muscle		Adipose tissue	
		F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO
c6,c9,t11-octadecatrienoic acid	TG	ND^{a}	1.72 ± 0.26^{b}	ND^{a}	1.38 ± 0.14^{b}	ND^{a}	1.26 ± 0.13^{b}	ND^{a}	1.94 ± 0.21^{b}
	PL	ND^{a}	3.80 ± 0.92b	ND^{a}	2.14 ± 0.44^{b}	ND^{a}	2.42 ± 0.23^{b}	ND	ND
Alpha-linolenic acid	TG	0.39 ± 0.07^{a}	3.92 ± 1.08^{b}	0.73 ± 0.04^{a}	5.93 ± 1.27^{b}	0.73 ± 0.05^{a}	4.05 ± 0.71^{b}	1.09 ± 0.04^{a}	5.96 ± 1.32^{b}
	PL	0.09 ± 0.02^{a}	0.47 ± 0.09^{b}	0.08 ± 0.002^{a}	0.29 ± 0.07^{b}	0.12 ± 0.005^{a}	0.45 ± 0.11^{b}	ND	ND
Punicic acid	TG	ND^{a}	4.69 ± 0.99^{b}	ND^{a}	1.38 ± 0.43^{b}	ND^{a}	1.38 ± 0.29^{b}	ND^{a}	1.44 ± 0.37^{b}
	PL	ND^{a}	3.18 ± 0.70b	ND^{a}	0.67 ± 0.10^{b}	ND^{a}	0.91 ± 0.28^{b}	ND	ND
Arachidonic acid	TG	0.36 ± 0.07^{a}	0.33 ± 0.05^{a}	0.14 ± 0.01^{a}	0.12 ± 0.02^{a}	0.15 ± 0.03^{a}	0.12 ± 0.03^{a}	0.08 ± 0.01^{a}	0.05 ± 0.01^{a}
	PL	10.85 ± 0.30^{a}	7.53 ± 0.82^{b}	26.14 ± 1.48^{a}	24.08 ± 1.08^{a}	13.04 ± 0.89^{a}	10.42 ± 1.41^{a}	ND	ND
Eicosapentaenoic acid	TG	Traces^{a}	0.02 ± 0.01^{b}	Traces^{a}	0.01 ± 0.001^{a}	Traces^{a}	0.01 ± 0.01^{b}	ND	ND
	PL	0.03 ± 0.002^{a}	0.50 ± 0.16^{b}	0.05 ± 0.01^{a}	0.47 ± 0.16^{b}	0.03 ± 0.004^{a}	0.29 ± 0.08^{b}	ND	ND
n-6 Docosapentaenoic acid	TG	0.06 ± 0.01^{a}	ND^{b}	0.01 ± 0.001^{a}	Traces^{b}	0.01 ± 0.004^{a}	ND^{b}	ND	ND
	PL	1.98 ± 0.17^{a}	0.20 ± 0.05^{b}	0.91 ± 0.06^{a}	0.09 ± 0.02^{b}	1.60 ± 0.19^{a}	0.16 ± 0.03^{b}	ND	ND
n-3 Docosapentaenoic acid	TG	0.04 ± 0.01^{a}	0.16 ± 0.02^{b}	0.01 ± 0.002^{a}	0.03 ± 0.005^{a}	0.02 ± 0.005^{a}	0.04 ± 0.01^{a}	ND^{a}	0.01 ± 0.01^{b}
	PL	0.40 ± 0.05^{a}	1.40 ± 0.15^{b}	0.32 ± 0.04^{a}	0.95 ± 0.09^{b}	0.86 ± 0.09^{a}	1.76 ± 0.16^{b}	ND	ND

Table 6.5. Fatty acid composition of triglycerides and phospholipids in the liver, heart, adipose tissue and muscle of hens fed with the experimental diets (continued)

% total fatty acids	Lipid fractions	Liver		Heart		Muscle		Adipose tissue	
		F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO	F-OL	FS-PSO
Docosahexaenoic acid	TG	0.11 ± 0.02^{a}	0.30 ± 0.02^{b}	0.02 ± 0.001^{a}	0.04 ± 0.004^{a}	0.04 ± 0.01^{a}	0.08 ± 0.02^{a}	ND^{a}	0.01 ± 0.01^{b}
	PL	3.80 ± 0.46^{a}	8.30 ± 0.84^{b}	0.59 ± 0.04^{a}	1.42 ± 0.11^{b}	4.13 ± 0.17^{a}	7.61 ± 0.32^{b}	ND	ND
Σ SFA	TG	22.55 ± 0.84^{a}	26.83 ± 1.43^{b}	15.99 ± 0.42^{a}	19.34 ± 0.56^{b}	19.03 ± 0.34^{a}	23.00 ± 1.61^{b}	15.18 ± 0.34^{a}	19.33 ± 0.74^{b}
	PL	41.46 ± 0.48^{a}	38.75 ± 1.03^{b}	43.27 ± 0.97^{a}	42.50 ± 0.77^{a}	35.43 ± 3.55^{a}	37.42 ± 0.61^{a}	ND	ND
Σ MUFA	TG	64.50 ± 0.81^{a}	30.98 ± 2.00^{b}	65.08 ± 0.41^{a}	36.17 ± 1.33^{b}	62.44 ± 0.59^{a}	39.07 ± 1.84^{b}	67.10 ± 0.18^{a}	34.16 ± 2.13^{b}
	PL	28.30 ± 0.79^{a}	13.19 ± 0.89^{b}	16.14 ± 0.38^{a}	6.18 ± 0.64^{b}	30.08 ± 6.38^{a}	7.08 ± 0.65^{b}	ND	ND
Σ n-6 PUFA	TG	12.32 ± 1.35^{a}	18.29 ± 0.35^{b}	18.09 ± 0.55^{a}	24.19 ± 0.68^{b}	17.44 ± 0.73^{a}	20.41 ± 0.79^{b}	16.56 ± 0.33^{a}	23.87 ± 0.62^{b}
	PL	25.82 ± 0.71^{a}	20.30 ± 1.20^{b}	39.43 ± 1.08^{a}	38.76 ± 0.86^{a}	29.88 ± 2.23^{a}	35.73 ± 0.89^{b}	ND	ND
Σ n-3 PUFA	TG	0.54 ± 0.11^{a}	4.52 ± 1.10^{b}	0.76 ± 0.04^{a}	6.05 ± 1.27^{b}	0.79 ± 0.07^{a}	4.20 ± 0.73^{b}	1.09 ± 0.04^{a}	5.98 ± 1.31^{b}
	PL	4.35 ± 0.53^{a}	10.97 ± 0.97^{b}	1.06 ± 0.09^{a}	3.25 ± 0.31^{b}	4.45 ± 0.72^{a}	10.23 ± 0.51^{b}	ND	ND
Σ CLA	TG	0.02 ± 0.01^{a}	9.99 ± 1.10^{b}	0.02 ± 0.002^{a}	9.12 ± 0.86^{b}	0.04 ± 0.004^{a}	8.79 ± 1.10^{b}	0.01 ± 0.005^{a}	10.23 ± 1.20^{b}
	PL	0.02 ± 0.002^{a}	8.40 ± 1.10^{b}	Traces^{a}	5.85 ± 0.48^{b}	0.03 ± 0.01^{a}	5.48 ± 0.50^{b}	ND	ND
Σ CLnA	TG	ND^{a}	8.25 ± 1.57^{b}	ND^{a}	4.15 ± 0.30^{b}	ND^{a}	3.59 ± 0.33^{b}	ND^{a}	4.72 ± 0.46^{b}
	PL	ND^{a}	7.84 ± 1.73^{b}	ND^{a}	3.00 ± 0.52^{b}	ND^{a}	3.75 ± 0.58^{b}	ND	ND

Data are means ± standard error of the means (n = 5). Values with no common superscript letters (a, b) are significantly different at $p < 0.05$, using two-sample t-test. F-OL: hens' feed supplemented with 10% (wt.) olive oil. FS-PSO: hens' feed supplemented with 7.8% (wt.) flaxseeds and 7% (wt.) pomegranate seed oil. "Traces" means detectable but too low to be quantified.TG: triglycerides; PL: phospholipids; ND: not detected; Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ n-6 PUFA: total omega-6 polyunsaturated fatty acids; Σ n-3 PUFA: total omega-3 polyunsaturated fatty acids; Σ CLA: total conjugated linoleic acids; Σ CLnA: total conjugated linolenic acids.

6.4. Discussion

The inclusion in hens' feed of flaxseeds, a common ingredient for the enrichment of eggs in n-3 PUFA, together with an oil rich in CLnA, as a precursor for the endogenous formation of CLA, is a dietary feeding strategy to advantageously modify the fatty acid composition of eggs. We assessed the enrichment by comparison with olive oil, which was incorporated in a diet low in ALA.

Egg production, as well as egg and yolk weights were unaffected by either dietary treatment or duration of feeding. However, egg weight, as well as yolk weight tended to increase with hens' age, as expected (Johnston & Gous, 2007). Consistent with our results, Bean and Leeson (2003) reported no significant effect of feeding a flaxseed-based diet on egg production, egg weight and yolk weight between 28 and 53 weeks of age. Kostogrys et al. (2017) indicated that using PSO to naturally enrich eggs with CLA and CLnA did not alter egg quality parameters, as well as organoleptic properties. In this study, the physico-chemical and sensory properties of the eggs were not analysed. However, eggs have been tested for regular consumption for 3 months by a panel of egg eaters. No adverse effects of enrichment on egg quality were reported (Ngo Njembe et al., 2021b).

The results obtained on the enrichment of eggs in n-3 PUFA, RmA and PunA are in agreement with our previous reported results and others (Kostogrys et al., 2017; Ngo Njembe et al., 2021a). The amount of ALA and DHA deposition in eggs through the inclusion of flaxseeds in the FS-PSO diet was significantly improved, while a lower level of ALA intake supplied by the F-OL diet led to the accumulation of much less ALA and DHA in eggs, with no variation with the duration of feeding. We have previously shown that changing the fatty acid profile of eggs in response to a dietary modification is a gradual process. When the hens were monitored individually, the steady-state deposition of RmA and a stable fatty

acid profile in the egg yolk were achieved within two weeks of feeding, for the laying hen to adapt to the enriched diet and reach a plateau of transfer of dietary fatty acids into developing ovarian follicles (Ngo Njembe et al., 2021a). In the present study, the inclusion of PSO resulted in a substantial accumulation of PunA and RmA in FS-PSO eggs. Interestingly, the stability of RmA enrichment in FS-PSO eggs was not achieved within one month of feeding. In addition, the upward trend observed over the feeding period implies that the maximal accumulation level of RmA in eggs might not have been totally reached, even after 26 weeks.

The incorporation of PSO in the feed of the hens also allowed the deposition to some extent of another CLA isomer (t9,t11-CLA), which probably derived from the metabolization of CLnA other than PunA contained in PSO. Tsuzuki and co-investigators (2006) reported that PunA and α-eleostearic acid are converted in RmA by a Δ13 saturation reaction carried out by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme, which is a novel enzyme recognizing conjugated trienoic acids or the enzyme active in the leukotriene B4 reductive pathway. Suzuki et al. (2006) detected t9,t11-CLA in the liver tissue and colonic mucosa of rats fed a diet containing *Catalpa ovata* seed oil, an oil rich in catalpic acid. Additionally, Schneider et al. (2013) showed that the CLA isomer produced from β-eleostearic acid and catalpic acid is t9,t11-CLA. These results indicate that the hen is able to efficiently convert various CLnA isomers with a Δ13 double bond.

Feeding PSO along with flaxseeds to hens increased the concentration of linoleic acid in FS-PSO eggs but decrease the level of arachidonic acid. The competition between n-6 PUFA and n-3 PUFA substrates, linoleic acid and ALA, respectively, to use the same enzymatic machinery for the bioconversion into longer chain PUFA is well known. The conversion of linoleic acid to arachidonic acid and ALA to EPA requires an alternating sequence of chain desaturation and elongation reactions catalyzed by Δ6- and Δ5-desaturases, and elongases. ALA is preferably metabolized over

linoleic acid by these enzymes (Kinsella, 1991). In birds consuming ALA, long-chain n-6-PUFA (arachidonic acid and n-6 DPA) decreased and were replaced by long-chain n-3 PUFA (EPA, n-3 DPA and DHA). On the other hand, RmA has been shown to undergo elongation and desaturation processes similar to those that occur with linoleic acid, maintaining the conjugated diene structure. Thus, RmA underwent a Δ6-desaturation leading to the formation of c6,c9,t11-CLnA. We failed to detect other long-chain conjugated diene derivatives. Likely, their levels were too low to accumulate in the yolk. Furthermore, cis-7, trans-9-hexadecadienoic acid found in FS-PSO eggs is probably a conjugated C16:2 metabolite derived from peroxisomal β-oxidation of RmA (Banni, 2002).

Concerning SFA and MUFA, the current results are consistent with other studies indicating that PunA and RmA have an inhibitory action on Δ9-desaturase (Choi, Park, Storkson, Pariza, & Ntambi, 2002; Kostogrys et al., 2017;Ngo Njembe et al., 2021a), leading to an increase in SFA. Although feeding with the F-OL diet significantly increased OA in eggs produced by hens in this group as compared to the FS-PSO group, the negative effect of PunA and RmA on MUFA accumulation could be observed through a significant decline in FS-PSO eggs over the course of the feeding period.

Similar to other monogastric animals, laying hens have a limited endogenous enzymatic ability to modify the structure of dietary fatty acids (Haug, Nyquist, Thomassen, Høstmark, & Ostbye, 2014). The level of fatty acids in the yolk is finite due to the total fat content of 30 to 33% and reaching a plateau of saturation or a stable state of accumulation for a fatty acid in the yolk is directly influenced by the nature and the concentration of dietary fatty acids. The current results showed that the stability of the fatty acid composition of the egg yolk for either the F-OL group or the FS-PSO group was achievable after one month of feeding. During post-absorptive metabolism, fatty acids are deposited in the egg yolk as TG for long-term energy storage, or as PL mobilized more rapidly

during embryonic tissue development (Speake, Murray, & Noble, 1998). The different dietary supplementations had no effect on the distribution of OA, ALA and DHA in the various lipid fractions. Moreover, PunA and RmA were mostly deposited in the TG fraction according to distribution patterns that were closer to that of OA than to ALA.

The fatty acid profiles of hens' tissues reflected those of the diets with significant levels of OA in the F-OL group, and ALA and PunA in the FS-PSO group. Similar to eggs, an accumulation of DHA and RmA, an increase in SFA and a decrease in MUFA related to the metabolization of ALA and PunA were observed in tissue lipids of the FS-PSO group. This suggests that the entire lipid metabolism in the hen was altered. As could be expected, the fatty acid composition of the TG and PL lipid classes in hens' tissues was similar to that of egg yolk. PunA and RmA in the hens' tissues were preferentially stored as an energy source in the TG fraction. This distribution pattern is also true for other food-borne CLnA, depending on their supply through the diet. Unlike its non-conjugated counterpart ALA, PunA was also deposited in appreciable amount in the PL fraction. Likewise, RmA and even more its metabolic derivative c6,c9,t11-CLnA were not only found at high levels in the TG fraction of tissue lipids, but were also incorporated into cell membrane phospholipids. This result is consistent with those of Banni and co-investigators, reporting in rats that RmA and its desaturation metabolite, the conjugated diene C18:3, are incorporated into liver phospholipids in a proportion ranging from 18% to 31% of their total amount. They also found that these two fatty acids were predominantly present in the phosphatidylcholine fraction (Banni et al., 2001). This last observation cannot be confirmed in our study, since we did not carry out the analysis of the different fractions of PL.

The inclusion of flaxseeds and PSO resulted in a decrease in arachidonic acid in the PL fraction. Belury and Kempa-Stecko (1997), when analysing the liver fatty acid composition of mice fed

with dietary CLA, reported that because CLA compete with linoleic acid for Δ6-desaturase, diet-induced changes in the composition of phospholipid fatty acids were related to the partial replacement of arachidonic acid by other fatty acids such as n-3 PUFA. In our study, it appears that membrane-bound arachidonic acid has been replaced primarily by long-chain n-3 PUFA, as well as by PunA, RmA and c6,c9,t11-CLnA. The different biological effects of PunA, RmA and their metabolites may be partly explained by their incorporation into membrane structures where they can affect cellular characteristics and physiological processes, including membrane fluidity and transmembrane receptor function.

6.5. Conclusion

This study was designed to evaluate the impact of different dietary fatty acid sources (olive oil vs. flaxseeds + PSO) on egg yolk deposition and fatty acid composition of four important tissues (liver, heart, muscle and adipose tissue) of the hen. Supplementation with flaxseeds and PSO altered the fatty acid composition of egg yolks, as well as tissues in hens. Dietary PunA was converted into RmA, and both fatty acids were incorporated into egg yolks and hens' tissues. Enrichment of the eggs with ALA, DHA, PunA and RmA was persistent, and a steady state of accumulation was achieved for most of the fatty acids after one month of feeding. In addition, fatty acids derived from RmA were also detected in eggs and hens' tissues. Taken together, these results indicate that combining flaxseeds with PSO is an effective way to produce eggs enriched with ALA, DHA, PunA and RmA and provide animal products with added nutritional value for human consumption.

6.6. References

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6.7. Additional data

Table 6.6. Composition of laying hens' diets

In percentage (%) of feed	**Standard feed** [a]	**F-OL diet**	**FF-PSO diet**	**Nutritional recommendations** [c]
Crude protein	16.0	15.03	16.10	max. 20
Fat	5.2	14.7	14.8	4 – 7
Crude fiber	5.0	4.5	6.4	max. 7
Calcium [b]	3.5	3.1	3.0	min. 3.5
Phosphorus	0.46	0.41	0.44	min. 0.31
Sodium	0.15	0.14	0.13	min. 0.12
Lysine	0.67	0.60	0.66	min. 0.65
Methionine	0.30	0.27	0.30	min. 0.3

[a] Ingredients: corn, wheat, sunflower meal, wheat gluten, calcium carbonate, palm oil, lecithin (soybean), sodium chloride, sodium bicarbonate.
Additives/kg: Vitamin A 10,000 IU; Vitamin D3 1500 IU; Vitamin E 30 mg; 25-Hydroxycholecalciferol 0.038 mg; Betaine anhydrous 650 mg; Ferrous sulfate, monohydrate, 55 mg; Anhydrous calcium iodate 1.80 mg; Cupric sulfate, pentahydrate, 15 mg; Manganous oxide, 100 mg; Zinc sulphate, monohydrate, 70 mg; Sodium selenite, 0.35 mg; Lutein 2.3 mg; Canthaxanthin 3.5 mg; Zeaxanthin 0.11 mg.

[b] The grit made up of a mixture of shellfish scales was given separately to the hens to increase the calcium intake.

[c] Ref.: Leroyer et al. Cahier technique : Produire des œufs biologiques en AB. ITAB, 2010.

Chapter 7

A three-month consumption of eggs enriched with ω-3, ω-5 and ω-7 polyunsaturated fatty acids significantly decreases the waist circumference of subjects at risk of developing metabolic syndrome

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Foreword

In the present study, eggs developed in Chapter 6 were consumed by volunteers during a set period of time and the health benefits were assessed. No specific preparation method was recommended for the eggs because, as presented in the additional data, omega-3 fatty acids, rumenic acid and punicic acid are stable during different culinary processes.

Abstract

Alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA), and punicic acid (PunA) are claimed to influence several physiological functions including insulin sensitivity, lipid metabolism and inflammatory processes. In this double-blind randomized controlled trial, we investigated the combined effect of ALA, DHA, RmA and PunA on subjects at risk of developing metabolic syndrome. Twenty-four women and men were randomly assigned to two groups. Each day, they consumed two eggs enriched with oleic acid (control group) or enriched with ALA, DHA, RmA, and PunA (test group) for 3 months. The waist circumference decreased significantly (-3.17 cm; $p < 0.001$) in the test group. There were no major changes in plasma insulin and blood glucose in the two groups. The dietary treatments had no significant effect on endothelial function as measured by peripheral arterial tonometry, although erythrocyte nitrosylated hemoglobin concentrations tended to decrease. The high consumption of eggs induced significant elevations in plasma low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol ($p < 0.001$), which did not result in any change in the LDL/HDL ratio in both groups. These results indicate that consumption of eggs enriched with ALA, DHA, RmA and PunA resulted in favorable changes in abdominal obesity without affecting other factors of the metabolic syndrome.

Keywords: Alpha-linolenic acid; Docosahexaenoic acid; Rumenic acid; Punicic acid; Enriched eggs; Metabolic syndrome; Waist circumference; Obesity

7.1. Introduction

Metabolic syndrome (MetS) encompasses a complex set of interrelated physiological and biochemical disorders, including disruption of lipid and glucose metabolism associated with vascular abnormalities and a pro-inflammatory state. Its most prominent components are abdominal obesity, hypertension, hyperglycaemia, hypertriglyceridemia and low level of high-density lipoprotein (HDL)-cholesterol (Kassi, Pervanidou, Kaltsas, & Chrousos, 2011). These metabolic disorders dramatically increase the risk of type 2 diabetes, coronary diseases, and stroke. The International Diabetes Federation indicates that people with MetS have a fivefold greater risk of developing type 2 diabetes, and that they are three times more likely to have a heart attack or stroke and twice as likely to die from it (Alberti, Zimmet, Shaw, & Grundy, 2005). The prevalence of MetS is estimated at around one quarter of the world's population. Although this estimate varies depending on the age, ethnicity and gender of the population studied, the metabolic alterations are aggravated by lifestyle, including inactivity and dietary factors, mainly fats and some types of carbohydrates, which are strongly suspected of inducing both their development and complications (Nolan, Carrick-Ranson, Stinear, Reading, & Dalleck, 2017; Saklayen, 2018).

There has been an increased interest over the past decade in understanding the metabolism of dietary lipids and their role in health. The special attention in fats has also come from the recognition that certain fatty acids are key regulators of gene expression and metabolic processes. In the worrisome context of the escalation of cardiovascular diseases (CVD) and type 2 diabetes, many researchers have studied the impact of some fatty acids on improving MetS factors.

Several follow-up studies reported that intakes of omega-3 polyunsaturated fatty acids (n-3 PUFA), including α-linolenic acid (ALA, *C18:3c9,c12,c15*) and its long-chain derivative,

docosahexaenoic acid (DHA, *C22:6c4,c7,c10,c13,c16,c19*), are strongly associated with a lower risk of MetS (Baik, Abbott, Curb, & Shin, 2010; Kim et al., 2016). Beneficial effects on the action of insulin have also been revealed by an inverse association found between the content of n-3 PUFA in the blood and insulin resistance (Nigam, Frasure-Smith, Lesperance, & Julien, 2009; Thorseng et al., 2009). A 3-month study in obese men indicated that supplementation with rumenic acid (RmA, *C18:2c9,t11*), an omega-7 conjugated linoleic acid (CLA) slightly decreased insulin sensitivity without altering serum lipids, glycaemia, body mass index (BMI) and body fat (Riserus, Vessby, Arnlov, & Basu, 2004). In contrast, Schmitt et al. (2006) reported a preponderant effect of RmA in reducing insulinemia and counteracting insulin resistance after 60 days in obese and diabetic patients receiving a diet based on products rich in ALA and DHA. This suggests that combining RmA with n-3 PUFA would lead to beneficial effects on glycaemic parameters. Furthermore, punicic acid (PunA, *C18:3c9,t11,c13*), an omega-5 conjugated linolenic acid (CLnA), given to mice with diet-induced obesity, prevented excess body fat and improved insulin sensitivity (Vroegrijk et al., 2011). Up to now, the combined effects of PunA, n-3 PUFA and RmA on obesity, serum lipids and glucose metabolism have not been established in both animals and humans.

In previous work, we have developed a method to enrich eggs with ALA, DHA, RmA and PunA through hen feeding (Ngo Njembe et al., 2021). The present study aimed at determining the effect of regular consumption of these eggs in adults at risk of MetS. Several key factors of the MetS were assessed, including glycosylated hemoglobin (HbA1c), insulin and fasting glucose parameters, blood pressure, abdominal obesity, lipids, and lipoproteins. We extended our study to the analysis of vascular health indicators. These include measurements of erythrocyte nitrosylated hemoglobin and endothelial function.

7.2. Materials and Methods

7.2.1. Study population

Participants were recruited from the population aged 35 to 75, with a waist circumference greater than 80 cm for women and 94 cm for men, and practicing fewer than two hours of physical activity per week. Eligible participants completed a telephone screening to attend a medical check-up scheduled two weeks prior to the start of the study, at the Center of Investigation in Clinical Nutrition (Louvain-la-Neuve, Belgium). Comorbidity assessment and medical history review were performed for all subjects who provided information on allergies, food intolerances, smoking, drug addiction and current medication use.

Exclusion criteria were: uncontrolled hypertension (> 160/100 mmHg), type 1 or type 2 diabetes (fasting blood sugar ≥ 126 mg/dl or HbA1c ≥ 6.5%), CVD or high risk of CVD (total cholesterol > 239 mg/dl, low-density lipoprotein (LDL)-cholesterol > 159 mg/dl, HDL-cholesterol < 40 mg/dl, triglycerides > 200 mg/dl or familial history of premature cardiovascular incident); be involved in an active weight loss program or have experienced weight loss of more than 5 kg in the past three months, receiving medication or have other health issues that might compromise compliance with the study interventions. Pregnant, lactating and perimenopausal women with symptoms, as well as postmenopausal women for less than six months were also excluded from participating. All selected participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki, and the Ethical Committee of the Cliniques universitaires Saint-Luc (Brussels, Belgium) approved the protocol. The trial is registered in the ClinicalTrials.gov database as NCT04583657.

7.2.2. Study design

Participants were randomly assigned in a 1:1 ratio to either a test group or a control group. Randomization was stratified by gender using a computer-generated list of random numbers prepared by an independent statistician. In double-blind design, the test group received daily two eggs enriched with ALA, DHA, RmA and PunA, while the control group participants were given two eggs enriched with oleic acid (OA). The eggs were meant to be eaten cooked, preferably at breakfast or lunch, for three consecutive months. All eggs were produced at the University Farm of UCLouvain (Corroy-le-Grand, Belgium). The fatty acid profile of the eggs (Table 7.1) was checked weekly as described previously (Ngo Njembe et al., 2021), to ensure it was constant throughout the study. An intervention staff strictly maintained blinding by packing the eggs in similar cardboard boxes on which a unique 3-letter code assigned to each participant was printed. Neither the assessors nor the participant knew which treatment the participant received. Boxes containing 16 eggs (2 additional eggs to prevent possible loss) were delivered to participants weekly. Upon delivery, uneaten eggs from the previous week were collected and participants responded to a questionnaire asking the occurrence, nature, severity and duration of potential adverse events, their link with the study, and whether participants had changed medication or taken a particular drug.

The three-month trial period included four medical visits: one at the start of the study (month 0 or baseline), when the subjects received the eggs for the first time, and three monthly visits (months 1, 2 and 3), the last of which ended the study. The primary endpoint chosen for the design of the study was the mean change in HbA1c from baseline to month 3. Other efficacy endpoints included obesity, serum lipids, insulin sensitivity, inflammation, and endothelial function. All assessments, except endothelial function, were performed at each monthly visit. The participants were requested not to change their habits regarding

physical activity, to eat fish no more than twice a week, and to abstain from dietary supplements of n-3 PUFA, CLA or CLnA during the trial commitment period. The study was conducted from June 2019 to March 2020.

Table 7.1. Composition of fatty acids, total lipid, and cholesterol content of eggs

	Control	**Test**
Fatty acids (mg/egg)		
Lauric acid (C12:0)	3.93 ± 0.08	3.95 ± 0.08
Myristic acid (C14:0)	9.47 ± 0.21	12.04 ± 0.28
Palmitic acid (C16:0)	993.91 ± 17.29	1008.68 ± 16.67
Palmitoleic acid (C16:1c9)	56.24 ± 2.00	51.03 ± 1.81
Stearic acid (C18:0)	344.47 ± 5.90	386.41 ± 5.52
Oleic acid (C18:1c9)	2689.32 ± 33.29	1115.25 ± 25.97
Cis-vaccenic acid (C18:1c11)	70.96 ± 1.25	35.58 ± 1.04
Linoleic acid (C18:2c9,c12)	543.01 ± 10.58	622.70 ± 13.30
Gamma-linolenic acid (C18:3c6,c9,c12)	3.52 ± 0.13	4.20 ± 0.13
Alpha-linolenic acid (C18:3c9,c12,c15)	14.31 ± 0.34	105.19 ± 4.04
Rumenic acid (C18:2c9,t11)	2.06 ± 0.21	595.16 ± 10.64
Punicic acid (C18:3c9,t11,c13)	ND	321.59 ± 9.25
Dihomo-gamma-linolenic acid (C20:3c8,c11,c14)	6.50 ± 0.13	7.55 ± 0.17
Arachidonic acid (C20:4c5,c8,c11,c14)	97.89 ± 1.46	62.40 ± 1.45
Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)	0.05 ± 0.02	2.58 ± 0.11
n-6 Docosapentaenoic acid (C22:5c4,c7,c10,c13,c16)	23.35 ± 0.70	4.47 ± 0.24
Docosahexaenoic acid (C22:6c4,c7,c10,c13,c16,c19)	37.72 ± 0.69	82.81 ± 1.89
Σ SFA	1378.63 ± 22.40	1445.70 ± 22.31
Σ MUFA	2833.56 ± 35.64	1221.60 ± 28.01
Σ n-6 PUFA	691.28 ± 119.13	716.03 ± 138.72
Σ n-3 PUFA	61.55 ± 1.02	230.16 ± 6.15
Total lipids (% by weight of fresh yolk)	31.08 ± 0.59	32.45 ± 0.44
Total cholesterol (mg/egg)	177.19 ± 5.40	182.26 ± 10.13

Values as "mean ± SEM". ND: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; n-6 PUFA: omega-6 polyunsaturated fatty acids; n-3 PUFA: omega-3 polyunsaturated fatty acids.

7.2.3. Dietary assessment

Prior to medical visit, the subjects performed a 3-day dietary record. Standard food servings and a food atlas (Yasar, Nicolas, & Dufourny, 2010) were used to quantify household measures. The macronutrient composition of the diets was assessed using the French Agency for Food, Environmental and Occupational Health Safety database (ANSES-CIQUAL).

7.2.4. Anthropometric measurements

Body weight was determined to the nearest 0.1 kg, height, waist and hip circumferences to the nearest 0.1 cm, in subjects wearing light indoor clothing and without shoes. The waist-to-hip ratio (WHR) was calculated from these measurements. BMI was calculated by dividing the body weight in kilograms by the square of the height in meters. Body fat and lean body mass were calculated using a Tanita SC-240 bioelectric impedance analyzer (Tanita Europe BV, Amsterdam, The Netherlands) and manufacturer's programmed equations.

7.2.5. Clinical investigations

Blood samples were obtained from subjects after a 10- to 12-h overnight fast. Triglycerides, HDL-, non-HDL- and LDL-cholesterol, glycaemia, insulinemia, homeostasis model assessment for insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), HbA1c, hemoglobin, hematocrit, erythrocytes, leucocytes, thrombocytes, high-sensitivity C-reactive protein (hs-CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT), urea, creatinine, estimated glomerular filtration rate (eGFR) and albumin were analyzed at the Clinique St Pierre (Ottignies, Belgium).

IL-6 and TNF-α assays were performed using Human IL-6 and TNF-α Quantikine HS ELISA kits (R&D Systems, Langley, UK) respectively, and oxidized LDL (ox-LDL) were measured employing a Mercodia oxidized LDL ELISA kit (Mercodia, Huissen, The Netherlands), according to manufacturer's instructions.

7.2.6. Measurement of nitrosylated hemoglobin and peripheral artery tonometry

On two occasions, at the first (month 0) and the last visit (month 3), the endothelial function was assessed by peripheral arterial tonometry and nitrosylated hemoglobin measurement.

Blood was drawn by venopuncture from the median cubital vein into a vacutainer tube containing EDTA (K2E, Vacutainer, BD-Plymouth, UK). A mixture of antioxidant solution (sodium ascorbate and N-acetylcysteine; final concentration, 5 mmol/l of both) was added into closed vacutainer using a Micro-Fine™ syringe prior the centrifugation to support the blood redox condition. The erythrocytes were collected after centrifugation (10 min, 800x*g*, at room temperature) from the bottom of the vacutainer tube into a 1 ml syringe and stored immediately at −80°C. The concentration of heme-(Fe II)-nitrosyl-hemoglobin (HbNO) was assessed in the erythrocyte samples using the low-temperature Electron Paramagnetic Spectroscopy (EPR). The EPR spectra were recorded by an X-band EPR spectrometer (EMX-micro) (Bruker Instruments Inc., Billerica, MA, USA) with the following settings: microwave frequency, ~ 9.35 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 0.7 mT at 77 K using an EPR quartz finger Dewar filled with liquid nitrogen. The erythrocyte concentration of the HbNO complex (5-coordinate α-heme-nitrosyl) was quantified from the intensity of the hyperfine components of the HbNO EPR signal (g-factor 2.01, Ahf = 16.8 G) after subtraction of the overlapping EPR signal of protein free radicals from the integral EPR spectrum of

frozen erythrocytes following the method developed previously (Lobysheva et al., 2017).

Endothelial vasodilation function was determined using the Endo-PAT 2000 device (Itamar Medical, Caesarea, Israel). Subjects were directed to rest in a quiet, dimly lit, temperature-controlled exam room. Two high-sensitive pneumatic probes (EndoPAT™, Itamar) were placed on the index fingers of the left and right hands, and plethysmographic signals in the index fingers were recorded throughout the test. A baseline record was performed for five minutes. For the 5-min occlusion phase, the blood pressure cuff placed on the right forearm was inflated to a supra-systolic pressure of 60 mmHg above the patient's systolic pressure or to 200 mmHg, depending on the greater value between the two. Complete cessation of blood flow to the hand was verified by the absence of a peripheral arterial tone (PAT) signal from the occluded arm. Then the cuff was abruptly deflated as quickly as possible to initiate the post-occlusion recording phase (5 min). The reactive hyperemia index (RHI) was calculated by the device as the ratio of the post-to-pre-occlusion PAT signal in the ischemic arm (right arm), relative to the same ratio in the control arm (left arm), and corrected for baseline vascular tone (Axtell, Gomari, & Cooke, 2010). Arterial stiffness was assessed by the augmentation index (AI), which was determined from the baseline resting pulse wave.

7.2.7. Statistics and data analysis

The study was designed to show a statistically significant 10% decrease in the primary endpoint (HbA1c) in the test group assuming a standard deviation of 0.8, using a two-sample t-test with 80% power and a 5% level of significance. PASS 14.0.7 software (NCSS, Kaysville, UT, USA) was used for the calculation based on 40 subjects per group and a dropout rate of 10%. Because of the lockdown measures intended to limit the spread of cases of

covid-19 contamination from March 2020 on, the trial had to be stopped after 24 subjects had completed the experimental period.

Statistical analyses were performed using the software systems SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and JMP Pro 15 SAS. Changes between groups (control and test) per visit (1, 2, 3 and 4) and between visits 1 and 4 for each group were analyzed using a linear mixed model for repeated measurements with subjects as random variable, and groups, visits, and their interaction as fixed independent variables. When the interaction was significant (p-value < 5%), pairwise comparisons were computed using t-tests followed by Bonferroni correction on selected combinations of groups and visits. When there was no significant interaction but the difference between groups or visits was significant, pairwise comparisons using Tukey's test were computed. If necessary, a logarithm to the base 10 was used to fulfill the assumptions of the mixed model. Variables that were not normally distributed after the logarithmic transformation were analyzed using non-parametric methods. For analyses of within-group differences, the Wilcoxon matched-pairs signed rank test was used. The Wilcoxon Mann-Whitney two-sample test was used for analyses of differences between groups.

7.3. Results

7.3.1. Dietary analysis and tolerance

Twenty-four subjects, 12 in each treatment group, were included in the study as shown in Figure 7.1 and Table 7.2 All participants completed the trial. Adherence to study was assessed based on the number of uneaten eggs and the dietary record provided by the participants. The estimate of eggs consumed over those prescribed was greater than 95% in all participants.

Table 7.2. Baseline characteristics of participants in control and test groups

	Control (*n* = 12)		**Test** (*n* = 12)		***p*-Value** [a]
	Mean ± SEM	**Range**	**Mean ± SEM**	**Range**	
Sex (women/men)	9/3	-	8/4	-	-
Age (years)	51.67 ± 2.39	42 – 71	45.58 ± 2.43	35 – 59	0.105
Body weight (kg)	76.23 ± 2.64	66.4 – 93.8	74.34 ± 4.18	59.1 – 98.1	0.402
BMI (kg/m^2)	25.98 ± 0.75	21.9 – 30.3	26.04 ± 1.20	20.3 – 35.6	0.402
Waist circumference (cm)	93.38 ± 2.69	82 – 111	92.21 ± 2.79	83 – 111	0.840
Hip circumference (cm)	106.38 ± 1.49	99 – 115	102.79 ± 2.91	92 – 132	0.296
WHR	0.88 ± 0.03	0.8 – 1.0	0.89 ± 0.02	0.8 – 1.1	0.564
Body fat (%)	33.48 ± 1.71	22.5 – 42.3	31.83 ± 2.24	18.1 – 48	0.624
Lean body mass (kg)	50.13 ± 2.56	42.7 – 66.6	49.81 ± 2.65	37.4 – 64.9	1.000
Hemoglobin (g/dl)	13.89 ± 0.34	12.2 – 15.5	14.18 ± 0.49	12 – 16.71	0.751
Hematocrit (%)	41.6 ± 0.89	36.1 – 46.4	42.20 ± 1.24	36.2 – 48.2	0.773
Red blood cells ($10^6/mm^3$)	4.66 ± 0.10	4.08 – 5.22	4.75 ± 0.16	3.93 – 5.66	0.977
White blood cells ($10^3/mm^3$)	5.28 ± 0.27	3.95 – 7.13	5.52 ± 0.26	3.93 – 7.27	0.507
Platelets ($10^3/mm^3$)	226 ± 14.09	144 – 301	222.17 ± 15.07	144 – 301	0.665
AST (UI/l)	18.83 ± 1.07	14 – 27	21.64 ± 2.13	14 – 35	0.477
ALT (UI/l)	17.25 ± 2.07	10 – 34	20.67 ± 2.69	10 – 44	0.247
GGT (UI/l)	15.50 ± 0.97	10 – 20	18.17 ± 2.52	9 – 36	0.664
Urea (mg/dl)	30.75 ± 1.50	20 – 38	33 ± 2.17	23 – 50	0.401
Creatininemia (mg/dl)	0.79 ± 0.02	0.67 – 0.93	0.86 ± 0.04	0.67 – 1.09	0.247
eGFR (ml/min)	92.42 ± 2.28	82 – 104	90.75 ± 3.35	73 – 112	0.623
Albumin (g/dl)	4.30 ± 0.09	3.90 – 4.90	4.31 ± 0.06	3.90 – 4.60	0.640
Total protein (g/dl)	6.94 ± 0.14	6 – 7.50	6.78 ± 0.12	6.20 – 7.30	0.271

n = number of subjects. [a] Differences between the two groups were analyzed using Wilcoxon Mann-Whitney two-sample test (significant at *p*-value < 0.05). BMI: body mass index; WHR: waist-to-hip ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltranspeptidase; eGFR: estimated glomerular filtration rate.

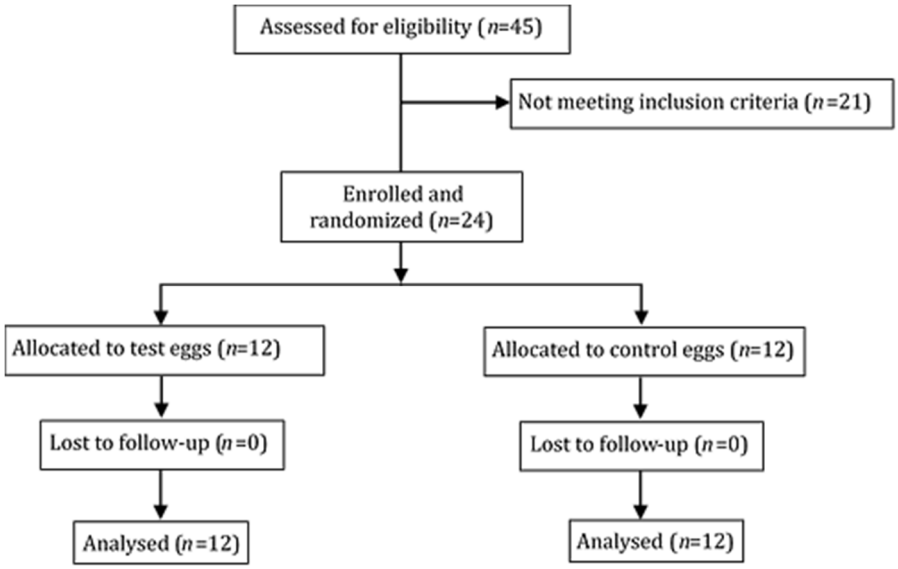

Figure 7.1. Recruitment flow diagram

Dietary analysis based on four 3-day food records, one completed before and the others completed during the study, showed that participants did not change their dietary intake with regard to energy, protein, carbohydrates, and fat (Table 7.3). Besides, the average daily intakes of energy and macronutrients did not differ significantly between groups. DHA intake increased in the test group. However, the difference in the DHA content of the control and the test eggs did not allow reaching a significant difference in the average daily intake of this n-3 PUFA between the two groups. In contrast, the consumption of approximately 1190 mg/day of RmA and 643 mg/day of PunA through the test eggs allowed a significant increase ($p < 0.001$) of their intakes by the test group. Subjects in both groups doubled their cholesterol intake during the study.

Participants experienced no adverse events except that two subjects in the test group reported mild nausea at some occasions. The following clinical chemistry variables: AST, ALT, GGT,

hemoglobin, hematocrit, erythrocytes, leucocytes, platelets, albumin, total serum proteins, urea, creatinine and eGFR were relatively constant and remained within normal ranges from the beginning to the end of the study in both groups.

7.3.2. Vital signs and anthropometrics

Heart rate, and systolic and diastolic blood pressures remained relatively constant in the two groups, without differences between them (Table 7.4). Body weight, BMI, waist circumference, and body composition were comparable across the two groups at the baseline (Table 7.2). When changes in these variables were analyzed over the course of the study, they displayed opposite patterns between the control group and the test group (Figure 7.2). However, the differences were not statistically significant for body weight, BMI, body fat and lean mass. The changes in waist circumference and in WHR were significant ($p < 0.05$) between the control and the test groups after two months of treatment. No significant difference was found within the control group from month 0 (baseline) to month 3, while waist circumference was reduced on average by 3.17 cm in the test group ($p < 0.001$, Table 7.4). To further explore the waist circumference response to dietary treatments, subjects were grouped by gender (Control group: females = 9 and males = 3; Test group, females = 8 and males = 4). No gender differences could be observed in any of the groups when performing this secondary analysis. The women and men in the control group showed a mean ± SEM reduction of 0.67 ± 1.02 and 0.01 ± 1.00 cm, respectively, while the women and men in the test group experienced a reduction in their waist circumference of 3.12 ± 1.41 and 3.25 ± 2.14 cm, respectively.

Table 7.3. Dietary intake of study participants based on the 3-day food record

	Months			
	0	**1**	**2**	**3**
Energy (kcal/day)				
Control	1865.2 ± 159.06 [a]	1811.92 ± 146.90 [a]	1805.75 ± 145.23 [a]	1818.33 ± 161.42 [a]
Test	1782.45 ± 100.32 [a]	1531.36 ± 81.53 [a]	1637.36 ± 105.59 [a]	1538.27 ± 137.10 [a]
Protein (% of daily energy)				
Control	16.00 ± 0.93 [a]	17.67 ± 1.24 [a]	17.92 ± 1.12 [a]	17.50 ± 0.72 [a]
Test	15.18 ± 1.23 [a]	17.27 ± 1.15 [a]	15.91 ± 0.94 [a]	18.55 ± 1.67 [a]
Carbohydrates (% of daily energy)				
Control	43.50 ± 1.92 [a]	38.67 ± 1.31 [a]	40.75 ± 1.72 [a]	40.33 ± 1.68 [a]
Test	38.27 ± 2.29 [a]	39.45 ± 2.18 [a]	36.64 ± 2.13 [a]	36.09 ± 2.02 [a]
Fiber (g/day)				
Control	21.4 ± 1.54 [a#]	20.00 ± 16.77 [a#]	19.17 ± 15.67 [a#]	20.58 ± 1.56 [a#]
Test	16.45 ± 1.77 [a#]	14.18 ± 1.32 [a#]	14.09 ± 1.47 [a#]	14.09 ± 135 [a#]
Lipids (% of daily energy)				
Control	35.62 ± 2.49 [a]	36.79 ± 1.74 [a]	36.19 ± 1.71 [a]	36.06 ± 1.73 [a]
Test	38.08 ± 2.19 [a]	36.58 ± 2.02 [a]	40.90 ± 2.17 [a]	40.09 ± 1.99 [a]
SFA (% of daily energy)				
Control	16.44 ± 1.25 [a]	14.80 ± 1.05 [a]	14.63 ± 1.22 [a]	14.25 ± 0.82 [a]
Test	15.22 ± 0.88 [a]	15.82 ± 1.42 [a]	17.59 ± 1.15 [a]	16.81 ± 0.83 [a]
MUFA (% of daily energy)				
Control	10.29 ± 0.95 [a]	12.70 ± 0.80 [b]	12.27 ± 0.80 [ab]	12.88 ± 0.74 [b]
Test	13.43 ± 1.51 [a]	11.40 ± 0.85 [a]	11.86 ± 1.43 [a]	11.09 ± 1.03 [a]

Table 7.3. Dietary intake of study participants based on the 3-day food record (continued)

	Months			
	0	**1**	**2**	**3**
PUFA (% of daily energy)				
Control	3.40 ± 0.36 [a]	4.80 ± 0.45 [b]	4.39 ± 0.31 [ab]	4.58 ± 0.34 [b]
Test	4.27 ± 0.62 [a]	5.02 ± 0.31 [a]	5.00 ± 0.44 [a]	5.56 ± 0.54 [a]
Alpha-linolenic acid (mg/day)				
Control	593.9 ± 132.63 [a]	930.58 ± 136.68 [a]	728.33 ± 103.98 [a]	679.17 ± 129.75 [a]
Test	697.81 ± 119.61 [a]	675 ± 87.35 [a]	629.73 ± 100.52 [a]	737.82 ± 168.48 [a]
Rumenic acid (mg/day)				
Control	125.9 ± 19.23 [a]	116.08 ± 28.92 [a##]	108.75 ± 19.82 [a##]	117.58 ± 17.81 [a##]
Test	92.64 ± 19.93 [a]	1167.27 ± 116.94 [b##]	1106.55 ± 131.88 [b##]	1187.73 ± 112.41 [b##]
Punicic acid (mg/day)				
Control	0.00 ± 0.00 [a]	0.00 ± 0.00 [a###]	0.00 ± 0.00 [a###]	0.00 ± 0.00 [a###]
Test	0.00 ± 0.00 [a]	643.18 ± 18.51 [b###]	643.18 ± 18.51 [b###]	643.18 ± 18.51 [b###]
Docosahexaenoic acid (mg/day)				
Control	148.20 ± 63.85 [a]	216.17 ± 51.55 [a]	230.42 ± 59.12 [a]	265.50 ± 126.67 [a]
Test	101.55 ± 37.62 [a]	189.64 ± 14.02 [ab]	278.27 ± 72.18 [b]	232.73 ± 34.41 [b]
Cholesterol (mg/day)				
Control	284.30 ± 38.36 [a]	621.50 ± 34.93 [b]	581.08 ± 19.91 [b]	593.5 ± 17.38 [b]
Test	262.45 ± 38.25 [a]	571 ± 21.43 [b]	589.09 ± 54.26 [b]	573.45 ± 23.66 [b]

Values as "mean ± SEM". Differences between the values were determined using t-test. Values in the same row with no common superscripts (a, b) are significantly different, $p < 0.05$. The difference between the two groups within the same month was significant at $p < 0.05$ (#), $p < 0.001$ (##) or $p < 0.0001$(###). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 7.4. Changes in blood pressure, anthropometric measurements, glycemic parameters and serum lipids between start and end of study

	Control (*n* = 12)			**Test** (*n* = 12)		
	Month 0	**Month 3**	**Δ, Month 3-0**	**Month 0**	**Month 3**	**Δ, Month 3-0**
Resting heart rate (beats/min)	68.33 ± 2.23	66.50 ± 2.22	−1.83 ± 2.22	66.25 ± 2.51	69.50 ± 2.24	3.25 ± 2.45
Systolic blood pressure (mmHg)	128.33 ± 4.80	122.33 ± 3.17	−6.00 ± 3.01	122.33 ± 4.76	118.17 ± 5.59	−4.17 ± 4.39
Diastolic blood pressure (mmHg)	78.50 ± 3.27	76.25 ± 1.85	−2.25 ± 2.58	77.08 ± 3.44	73.33 ± 3.57	−3.75 ± 2.45
Weight (kg)	76.23 ± 2.64	76.32 ± 2.76	0.09 ± 0.58	74.34 ± 4.18	73.48 ± 4.01	−0.86 ± 0.58
BMI (kg/m^2)	25.98 ± 0.75	26.01 ± 0.79	0.03 ± 0.19	26.04 ± 1.20	25.78 ± 1.16	−0.26 ± 0.19
Waist circumference (cm)[a]	93.38 ± 2.69	93.88 ± 2.69	0.50 ± 0.79	92.21 ± 2.79	89.04 ± 2.60	−3.17 ± 1.12**
Hip circumference (cm)	106.38 ± 1.49	105.63 ± 1.44	−0.75 ± 0.53	102.79 ± 2.91	101.83 ± 2.57	−0.96 ± 1.11
WHR	0.88 ± 0.03	0.89 ± 0.02	0.01 ± 0.01	0.90 ± 0.02	0.88 ± 0.03	−0.02 ± 0.01
Body fat (%)	33.48 ± 1.71	35.62 ± 2.30	2.14 ± 1.95	31.83 ± 2.24	29.48 ± 2.64	−2.35 ±1.37
Lean mass (kg)	50.13 ± 2.56	49.08 ± 2.43	−1.04 ± 1.64	49.81 ± 2.65	51.54 ± 3.00	1.73 ± 1.44
Fasting blood glucose (mg/dl)[a]	86.75 ± 1.44	86.42 ± 2.90	−0.33 ± 2.43	87.25 ± 2.69	89.42 ± 2.42	2.17 ± 2.77
Fasting insulin (mUI/ml)[a]	6.47 ± 0.66	5.76 ± 0.54	−0.71 ± 0.48	6.24 ± 0.82	7.38 ± 1.00	1.13 ± 0.90
HOMA-IR	1.40 ± 0.16	1.25 ± 0.14	−0.15 ± 0.12	1.38 ± 0.20	1.68 ± 0.27	0.30 ± 0.25
QUICKI	0.37 ± 0.03	0.38 ± 0.01	0.01 ± 0.004	0.38 ± 0.04	0.36 ± 0.01	−0.01 ± 0.01
HbA1c (mmol/mol)[a]	33.33 ± 1.00	34.00 ± 0.82	0.67 ± 0.31	33.33 ± 1.05	33.75 ± 1.02	0.42 ± 0.29

Table 7.4. Changes in blood pressure, anthropometric measurements, glycemic parameters and serum lipids between start and end of study (continued)

	Control (*n* = 12)			**Test** (*n* = 12)		
	Month 0	**Month 3**	**Δ, Month 3-0**	**Month 0**		**Month 0**
Triglycerides (mg/dl)	70.17 ± 5.74	70.83 ± 6.80	0.67 ± 7.29	77.25 ± 9.98	79.17 ± 11.82	1.92 ± 9.33
Total cholesterol (mg/dl)	184.67 ± 7.21	193.50 ± 7.95	8.83 ± 4.44**	170.50 ± 6.66	192.83 ± 6.67	22.33 ± 5.46**
LDL-cholesterol (mg/dl)	112.67 ± 6.39	118.08 ± 7.88	5.42 ± 3.60**	100.00 ± 6.29	116.00 ± 7.06	16.00 ± 4.51**
HDL-cholesterol (mg/dl)	57.92 ± 2.87	61.25 ± 2.92	3.33 ± 1.29**	55.08 ± 2.70	61.00 ± 3.36	5.92 ± 1.64**
LDL/HDL	1.99 ± 0.14	1.97 ± 0.15	−0.03 ± 0.05	1.90 ± 0.19	2.01 ± 0.20	0.10 ± 0.07
non-HDL-cholesterol (mg/dl)	126.75 ± 6.66	132.25 ± 7.38	5.50 ± 3.73**	115.42 ± 8.09	131.83 ± 8.28	16.42 ± 4.53**

n = number of subjects. Δ: change between the two months. Values as "mean ± SEM". BMI: body mass index; WHR: waist-to-hip ratio; HOMA-IR: homeostasis model assessment for insulin resistance; QUICKI: quantitative insulin sensitivity check index; HbA1c: glycosylated hemoglobin; LDL: low-density lipoprotein; HDL: high-density lipoprotein. [a] Variables were logarithmically transformed prior to paired t-test within groups. (**) Significant change from month 0 to 3, $p < 0.001$.

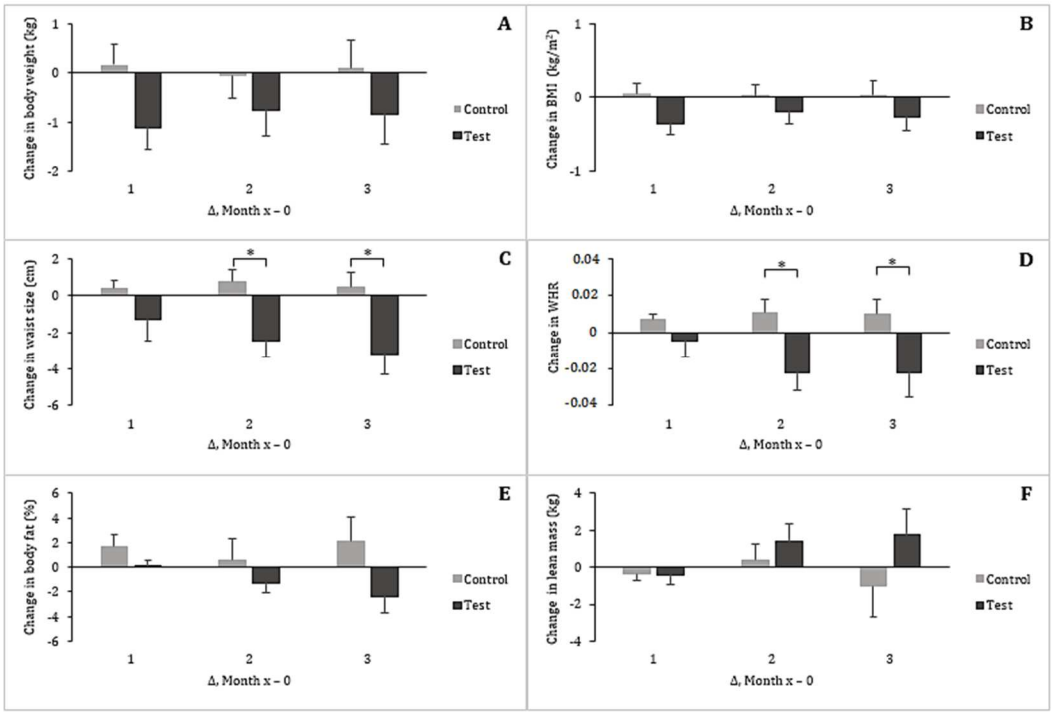

Figure 7.2. Changes (Δ) in body weight (**A**), body mass index (BMI) (**B**), waist circumference (**C**), waist-to-hip ratio (WHR) (**D**), percentage of body fat (**E**) and lean body mass (**F**) in men and women daily consuming two eggs enriched in oleic acid (control group) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) (test group), from the start of the study (Month 0) to months 1, 2 and 3 (Months x). Mean (± SEM). (*) for significant differences between control group and test group at $p < 0.05$, analyzed using t-test.

7.3.3. Insulin and glucose metabolism

Blood glucose and insulin levels did not differ in the two groups at month 3 compared to month 0. HOMA-IR and QUICKI were also not affected by the different treatments (Table 7.4). HbA1c levels increased significantly from month 0 to month 1 ($p < 0.01$) in the control group and from month 0 to months 1 and 2 ($p < 0.01$ and $p < 0.05$, respectively) in the test group, and then returned at month 3 to levels similar to those at month 0 (Figure 7.3). There was no significant difference between the two groups regarding values for the same month.

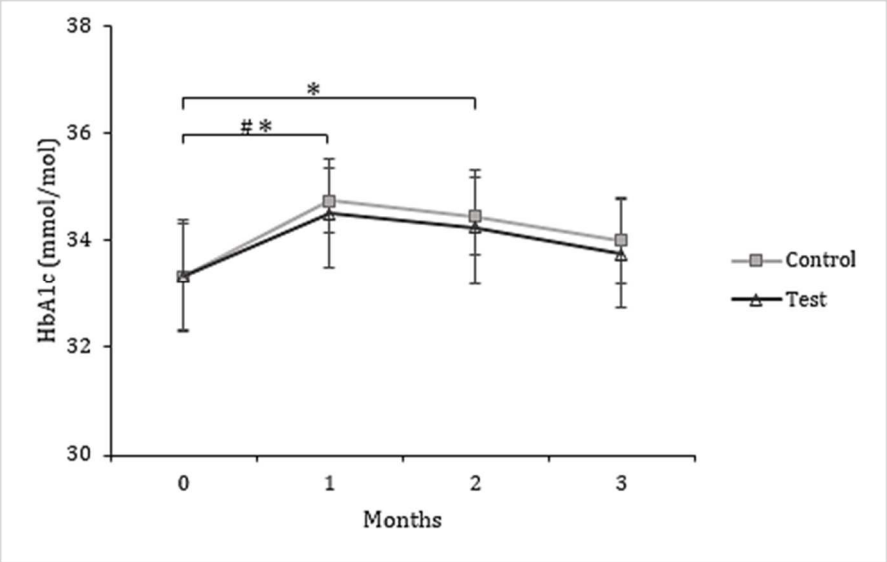

Figure 7.3. Glycosylated hemoglobin (HbA1c) in response to the daily consumption of two eggs enriched in oleic acid (OA) by the control group (□) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) by the test group (Δ), for 3 months. Mean (± SEM). Variables were logarithmically transformed prior to paired t-test, $p < 0.05$. (#) Significant difference compared to month 0 within the control group; (*) Significant difference compared to month 0 within the test group.

7.3.4. Serum triglycerides and lipoproteins

Changes in serum triglycerides were very small throughout the study and did not differ among treatment groups (Table 7.4). Total cholesterol concentrations were significantly increased in the two groups ($p < 0.001$), due to a significant increase in HDL and non-HDL-cholesterol ($p < 0.001$ in both groups) (Table 7.4, Figure 7.4). No difference between the groups was found. Analysis of within-group profiles showed that seven subjects in the control group had normal total cholesterol levels (< 190 mg/dl) at the start of the study. Five subjects remained within normal levels, while the other two had an increase in total cholesterol above normal level by the first month of treatment. One subject in the control group with a high level at month 0 had a decrease in total cholesterol to a normal level at month 3. Concerning the test group, ten subjects had normal total cholesterol levels at month 0. Four of them exceeded the normal limit (two from month 1 and two at month 3), whereas two other subjects experienced an increase in total cholesterol above normal at months 1 and 2, then a decrease to normal levels at month 3.

7.3.5. Inflammation and oxidative stress

At month 0, there were no significant differences in plasma inflammatory markers between the groups, except that hs-CRP was significantly lower ($p < 0.05$) in the test group (Table 7.5). Both treatments had no effect on IL-6 and TNF-α levels. There was a within-group trend toward an increase in hs-CRP ($p < 0.08$) in the test group, while no change was observed in the control group. Plasma ox-LDL levels increased in both groups at month 3 compared to month 0. However, the increase was statistically significant only in the test group ($p < 0.05$). There was no statistically significant difference between the groups at months 0 and 3.

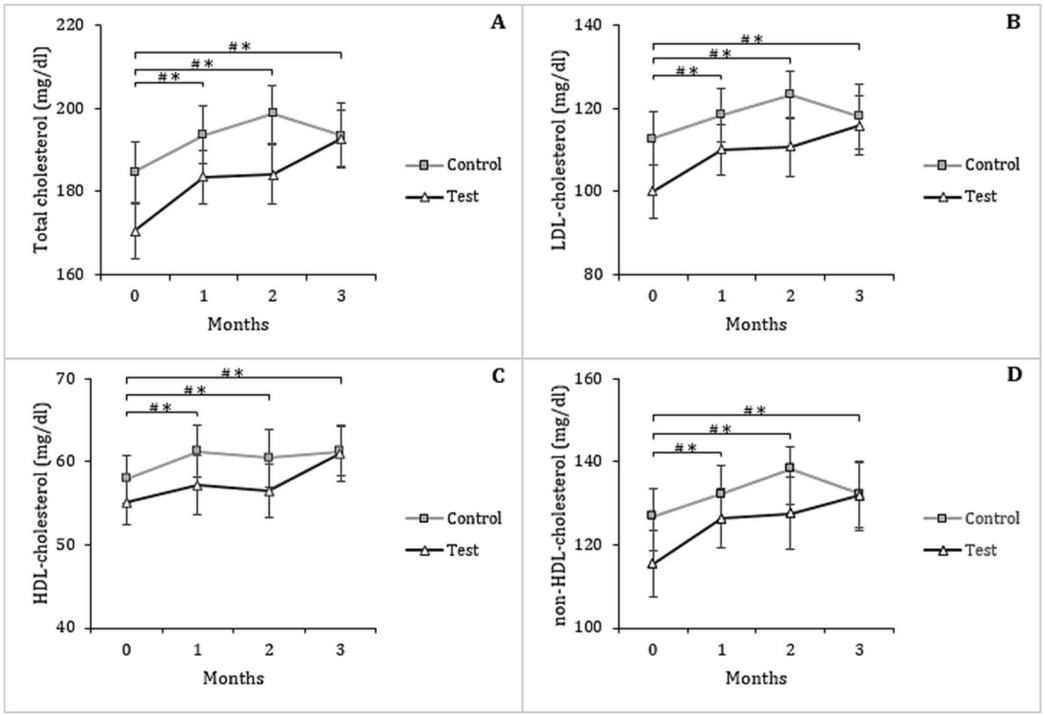

Figure 7.4. Total cholesterol (**A**), low-density lipoprotein (LDL)-cholesterol (**B**), high-density lipoprotein (HDL)-cholesterol (**C**) and non-HDL-cholesterol (**D**) in response to the daily consumption of two eggs enriched in oleic acid (OA) by the control group (□) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) by the test group (Δ) during 3 months. Mean (± SEM). Paired t-test, $p < 0.001$. (#) Significant difference compared to month 0 within the control group; (*) Significant difference compared to month 0 within the test group.

Table 7.5. Changes in inflammation, oxidative stress and vascular health parameters between start and end of study

	Control (*n*=12)			**Test** (*n*=12)		
	Month 0	**Month 3**	**Δ, Month 3-0**	**Month 0**	**Month 3**	**Δ, Month 3-0**
hs-CRP (mg/dl)	0.23 ± 0.06#	0.19 ± 0.04	–0.03 ± 0.06	0.09 ± 0.02#	0.21 ± 0.06	0.11 ± 0.06
IL-6 (pg/ml)	1.29 ± 0.21	1.29 ± 0.24	0.00 ± 0.27	1.20 ± 0.16	1.69 ± 0.36	0.49 ± 0.36
TNF-α (pg/ml)	0.87 ± 0.08	0.86 ± 0.07	–0.01 ± 0.04	0.79 ± 0.05	0.85 ± 0.05	0.06 ± 0.03
Ox-LDL (U/l)	55.66 ± 4.14	59.13 ± 3.75	3.47 ± 3.51	51.89 ± 4.29*	63.06 ± 3.94 *	11.16 ± 9.81
HbNO (nmol/l)	102.50 ± 10.37	78.44 ± 13.59	–24.06 ± 17.48	110.42 ± 18.29	83.86 ± 16.39	–26.56 ± 14.33
RHI	2.30 ± 0.18	2.17 ± 0.17	–0.18 ± 0.20	2.32 ± 0.19	1.98 ± 0.19	–0.34 ± 0.12
LnRHI	0.81 ± 0.08	0.74 ± 0.09	–0.09 ± 0.11	0.81 ± 0.08	0.63 ± 0.10	–0.17 ± 0.06
AI (%)	18.74 ± 6.20	20.50 ± 8.12	1.76 ± 4.33	12.60 ± 6.33	14.94 ± 5.66	2.33 ± 2.66
AI@75bpm (%)	8.92 ± 6.21	12.95 ± 9.01	4.03 ± 5.39	4.64 ± 5.66	7.34 ± 5.22	2.69 ± 2.53

n = number of subjects. Δ: change between the two months. Values as "mean ± SEM". (*) Significant difference within groups analyzed using Wilcoxon matched-paired signed rank test, $p < 0.05$. (#) Significant difference in the same month between the two groups analyzed using Wilcoxon Mann-Whitney two-sample test, $p < 0.05$. hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; Ox-LDL: oxidized low-density lipoprotein; HbNO: nitrosylated hemoglobin; RHI: reactive hyperemia index; AI: augmentation index; AI@75bpm: augmentation index adjusted to a heart rate of 75 beats per minute.

7.3.6. Erythrocyte level of nitrosylated hemoglobin and vascular endothelial function

As shown in Table 7.5, erythrocyte HbNO concentrations decreased in both study groups at month 3 (-24.06 nmol/l in the control group and -26.56 nmol/l in the test group). However, these differences were not statistically significant. The reactive hyperemia indexes (RHI and LnRHI) did not differ among groups and throughout the study. The AI measurement for arterial stiffness was not significantly altered by either condition tested.

7.4. Discussion

In this double-blind randomized controlled parallel-group trial, we examined the effect of combined supplementation of ALA, DHA, RmA, and PunA on MetS components in healthy women and men, but with a high propensity to develop a MetS. OA was chosen as the control for its benefits in the management of CVD (Aparicio-Soto, Sánchez-Hidalgo, Rosillo, Castejón, & Alarcón-de-la-Lastra, 2016). Its positive effects include lowering LDL-cholesterol and increasing HDL-cholesterol (Zambon et al., 1999), and it may also help in good control of hypertriglyceridemia (Giron, Sanchez, Hortelano, Periago, & Suarez, 1999). Chicken egg was of particular interest as a dietary carrier for supplementation since it is relatively high in lipids and has great culinary versatility. The good compliance and the zero dropout rate observed during the study indicate that both control and test eggs were well tolerated by the participants.

One of the findings of this study is that the waist circumference decreased significantly in the subjects given the test eggs, while body weight, percent of body fat and lean mass were relatively unchanged (Figure 7.2). This suggests changes in body fat distribution and a positive effect of these eggs on abdominal obesity. In the placebo-controlled, randomized clinical trial by Defina et al. (2010) where overweight and obese individuals

received n-3 PUFA supplementation (3 g/day for 6 months), no difference in weight and body composition was observed compared to the placebo group. These results were confirmed by subsequent studies in subjects with CVD and type 2 diabetes (Crochemore, Souza, de Souza, & Rosado, 2012; Jafari Salim, Alizadeh, Djalali, Nematipour, & Hassan Javanbakht, 2017). In contrast, multiple studies showed convincing effects of CLA on reducing weight and body fat in overweight and obese humans (Blankson et al., 2000; Gaullier et al., 2007; Smedman & Vessby, 2001). The isomers of CLA that have been the most investigated so far are RmA and t10,c12-CLA (*C18:2t10,c12*). In a meta-analysis examining the effectiveness of CLA in reducing body fat, Whigham et al. (2007) highlighted the low number of human studies conducted with a single isomer. The few existing interventions mostly agree that RmA does not have significant effect on body composition in humans (Riserus et al., 2004; Sluijs, Plantinga, de Roos, Mennen, & Bots, 2010). In agreement with this, numerous animal and some human studies have shown that of the two tested isomers of CLA, t10,c12-CLA specifically is responsible for the anti-obesity effects (Hargrave et al., 2002; Kennedy et al., 2010; Park, Storkson, Albright, Liu, & Pariza, 1999). The effects observed in the present study could not be attributed to t10,c12-CLA, as it was not identified in the test eggs. Similar to our results, a patented lipid-based formulation containing PunA induced a reduction of waist circumference of 1.05 cm without change in body weight, body fat and muscle mass. Unfortunately, no indication of the amount of PunA used was provided (Peláez-Jaramillo et al., 2020).

Koba et al. (2007) showed that PunA administered to mice induced a reduction in adipose tissue weight accompanied by an increased in carnitine-palmitoyltransferase activity in the liver and brown adipose tissue. The authors stated that the anti-obesity effect of PunA could be in part due to the stimulation of β-oxidation of fatty acids. Furthermore, Lai et al. (2012) found in 3T3-L1 cells that PunA decreased adipogenesis and preadipocyte

differentiation by down-regulating the levels of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein (C/EBP)β and C/EBPδ, as well as fatty acid synthase, a key enzyme in lipogenesis.

Fasting blood sugar and insulin remained unchanged in both groups throughout the study, as did HOMA and QUICKI since these indices are derived from blood sugar and insulinemia values. The HbA1c value provides information about the average concentration of glucose in the blood over the 2 to 3 months preceding the test. Surprisingly, the levels of HbA1c increased after one month of treatment before returning to baseline values at the end of the study (Figure 7.3). The reason of this transient modulation has not been identified. Since blood sugar, insulin and HbA1c were globally not affected between the start and the end of the study, we can state that the combined supplementation of ALA, DHA, RmA and PunA through egg consumption for 3 months has neither diabetogenic nor diabeto-mitigating effects.

One whole egg provides on average 177.19 mg (control egg) or 182.26 mg (test egg) of cholesterol (Table 7.1). Consequently, both groups reported a significant elevation in dietary cholesterol during the study period. The high blood cholesterol levels observed in both groups might therefore be attributed to the eggs consumed. Contradictorily, Fuller et al. (2015), in a 3-month study in overweight or obese people with prediabetes or type 2 diabetes, found no significant difference in the change in triglycerides, total, LDL- and HDL-cholesterol between people consuming a high-egg diet (≥ 12 eggs/week) compared with those consuming a low-egg diet (< 2 eggs/week). The same research group subsequently showed that the high-egg diet over a 12-month period produced no adverse effects on cardiovascular risk factors including triglycerides, total, LDL- and HDL-cholesterol, inflammatory markers, oxidative stress and glycaemia measurements (Fuller et al., 2018).

Herron et al. (2003) classified healthy people on the basis of their response to prolonged consumption of high dietary cholesterol. Hypo-responders, who experienced an increase in total cholesterol of < 0.05 mmol/l for each additional 100 mg of dietary cholesterol consumed, were 62.5%, whereas 37.5% experienced an increase in total cholesterol ≥ 0.41 mmol/l for each additional 100 mg and were considered hyper-responders. In the present study, 50% of the cohort exhibited a hyper-response to dietary cholesterol. Studies indicated that dietary cholesterol intake from eggs significantly increases both serum LDL- and HDL-cholesterol, resulting in only a marginal change in the LDL-/HDL-cholesterol ratio (Berger, Raman, Vishwanathan, Jacques, & Johnson, 2015; Blesso & Fernandez, 2018; Herron et al., 2003). This is consistent with our findings. Indeed, the LDL-/HDL-cholesterol ratio did not change during the 3-month study and remained ≤ 2.01.

Historically, elevated LDL-cholesterol has been associated with an increased risk of CVD. However, non-HDL-cholesterol appears to be a better predictor for atherosclerotic vascular events (Moriyama & Takahashi, 2016). Non-HDL-cholesterol is the total amount of lipoproteins containing apolipoprotein B (apoB), including very low-density lipoproteins and their metabolic remnants, intermediate density lipoproteins and chylomicrons. These lipoproteins can participate in atherogenesis by entering and getting trapped in the intima of the arterial wall (Arsenault et al., 2009; Varbo et al., 2013). The Multinational Cardiovascular Risk Consortium recently published an analytical tool to predict the long-term risk of CVD based on non-HDL-cholesterol levels. The reference value (hazard ratio, HR = 1.0) associated with the lowest risk of cardiovascular disease was set at 2.6 mmol/l or 100 mg/dl non-HDL-cholesterol in women and men. For individuals with the highest non-HDL-cholesterol levels (≥ 220 mg/dl; HR= 1.9 in women and 2.3 in men), the CVD event rate over 30 years was predicted to be approximately three-to-four-times higher than in those with the lowest non-HDL-cholesterol (< 100 mg/dl)

(Brunner et al., 2019). According to this assessment model, the HR value between the start and the end of the present study was slightly increased (from 1.2 to 1.3) in both groups, suggesting that the increase in non-HDL-cholesterol seen during the study did not provide a worrisome additional risk on the incidence of CVD in the subjects.

Another indicator of CVD risk is ox-LDL, which are generally considered to be proatherogenic. Elevated ox-LDL levels are reported to be strongly positively linked to both atherosclerosis and inflammatory markers, including CRP, TNF-α and IL-6 (Caparević, Kostić, Ilic, Stojanović, & Ivanović, 2006; Hulthe & Fagerberg, 2002). In our study, the test group treated with ALA, DHA, RmA and PunA enriched eggs experienced a moderate but significant increase in plasma ox-LDL levels (from 51.89 to 63.06 U/l, $p < 0.05$). Currently, reference ranges for ox-LDL have not been established. Based on a prospective observational study of an apparently healthy and non-MetS population, a threshold of ox-LDL < 60 U/l has been defined for people at low risk of developing MetS. A range of 60 to 69 U/l would characterize people at relatively moderate risk, while a cut-off ≥ 70 U/l would indicate a high risk (Holvoet, Lee, Steffes, Gross, & Jacobs, 2008). There was no change in inflammatory markers TNF-α and IL-6 in the test group, although a non-significant upward trend in hs-CRP was seen, suggesting the development of low-grade inflammation already observed in the control group since the start of the study.

In accordance with our results, previous studies have reported that relatively high levels of PUFA in the diet increased LDL particles susceptibility to lipid peroxidation (Di Nunzio, Valli, & Bordoni, 2016; Jenkinson, Franklin, Wahle, & Duthie, 1999; Reaven & Witztum, 1996). The type and the amount of fat in the diet influence the fatty acid composition of lipoproteins and cell membranes, and may therefore affect the sensitivity of LDL and cells to oxidative damage. The more unsaturated the fatty acid, the more easily it oxidizes. Therefore, OA is less sensitive to oxidation

compared with ALA, DHA, RmA and PunA (Tsimikas et al., 1999). If the latter are abundant in the LDL particles, they will promote the formation of ox-LDL. Yang et al. (2009) comparing the oxidative stabilities of CLnA and CLA with their corresponding non-conjugated counterparts, ALA and linoleic acid, found that CLnA were the most unstable, followed by CLA, ALA and linoleic acid, in descending order. Unsurprisingly, DHA has also been found to enhance the susceptibility of cells to oxidative stress (Di Nunzio et al., 2016).

In addition, dietary fatty acids contribute to the pro-oxidant activity of arterial wall cells, since the fatty acid composition of the cell membrane influences cellular formation of reactive oxygen species (ROS) (Reaven & Witztum, 1996). The increased production of ROS impairs nitric oxide (NO) bioavailability and prevents it from inducing vascular smooth muscle relaxation, which may lead to endothelial dysfunction. After a 3-month consumption of eggs enriched with mono- or poly-unsaturated fatty acids, erythrocyte HbNO levels were slightly reduced, indicating vascular oxidative stress and a reduction of NO bioavailability (Dei Zotti, Verdoy, Brusa, Lobysheva, & Balligand, 2020). This is consistent with the weakly elevated hs-CRP (around 0.20 mg/dl) and the increase in ox-LDL seen in the two groups at month 3. High cholesterol levels have also been found to decrease the production of endothelial NO (Feron, Dessy, Moniotte, Desager, & Balligand, 1999). As the erythrocyte level of HbNO was shown to be a surrogate index of the endothelial NO production (Dei Zotti, Lobysheva, & Balligand, 2018), this reduction of NO production was manifested by a decrease in HbNO levels, and was consistent with the observed cholesterol increase in both groups. However, the moderate decrease of the level of HbNO was not associated with impairment of the reactive hyperemia blood flow response assessed by peripheral arterial tonometry, likely due to the reduced number of subjects and the fact that peripheral arterial tonometry reflects more than an NO-dependent control.

7.5. Conclusion

This study, to our knowledge, is the first exploring the effects of eggs enriched with ALA, DHA, RmA and PunA on some metabolic parameters. The findings indicate that the consumption of these eggs during three months by subjects at high risk of MetS leads to a significant reduction in abdominal obesity, without improving other components of MetS including glycaemia-associated parameters. Although polyunsaturated fatty acids are expected to reduce the risk of CVD, they are prone to lipid peroxidation, and together with high cholesterol levels, may alter some markers of vascular oxidative stress. A limitation of this study is the small sample size, which may have prevented the emergence of certain differences between the two conditions of the study. Relevant outcomes (abdominal obesity, ox-LDL, HbNO), as well as their durability, deserve to be further studied on a larger sample and over a longer time frame. Another limitation is that to aid adherence, no strict dietary advice has been given regarding egg consumption. The participants did not change their eating behavior during the study period and the nutritional intake of cholesterol was not balanced. For future studies, prescribed diets or dietary guidance should be provided to participants in order to balance the cholesterol intake associated with egg consumption.

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7.6. References

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7.7. Additional data

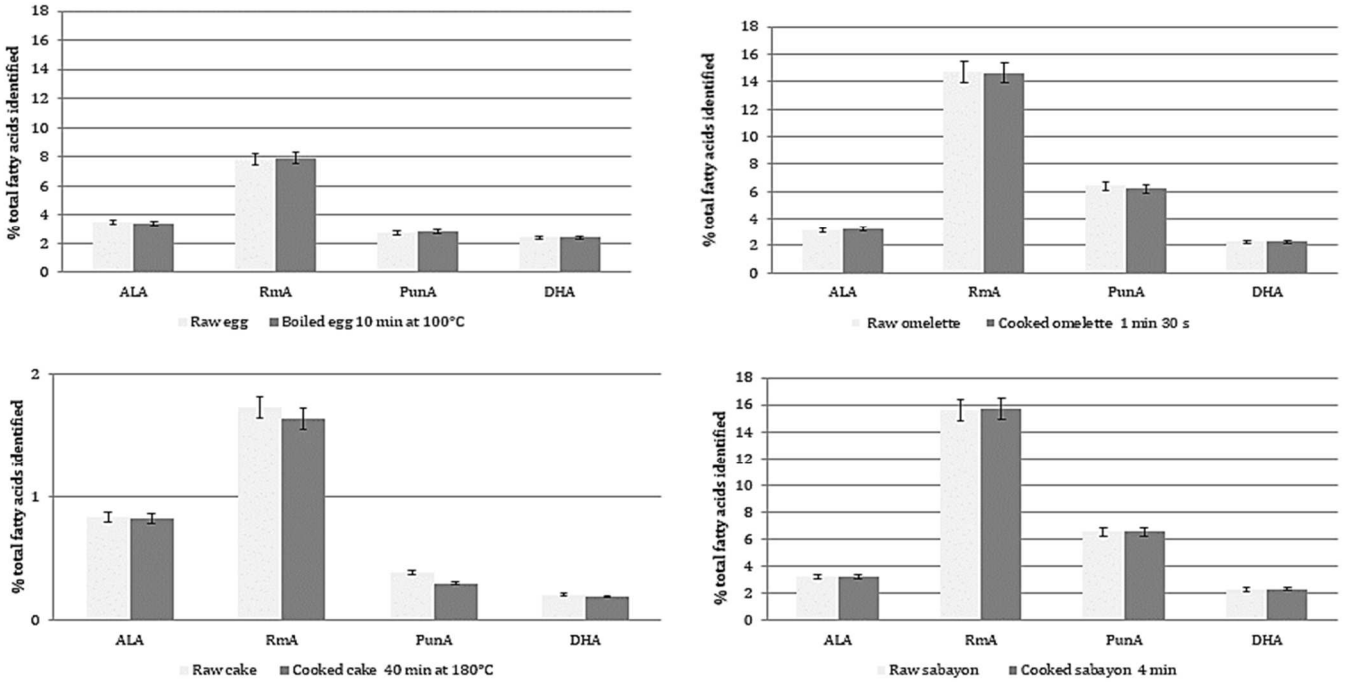

Figure 7.5. Stability of the content of α-linolenic acid (ALA), rumenic acid (RmA), punicic acid (PunA) and docosahexaenoic acid (DHA) in eggs during different culinary processes

Chapter 8

General discussion

CLA are provided to humans by the diet. However, the levels found in the principal dietary sources, which are dairy products and ruminant meat, rarely exceed 2% of total fatty acids. Besides, current nutritional recommendations call for reducing the consumption of animal fat, as well as ruminant meat. As a result, health-conscious consumers are opting for low-fat dairy products and choosing more foods derived from poultry. Chicken eggs contain relatively low CLA concentrations (approximately 2 mg CLA/egg). Developing eggs enriched with CLA may be an alternative way to increase CLA intake and provide health-promoting fatty acids to consumers. On the other hand, CLnA are present in large amounts in some plant seeds, such those of *Ricinodendron heudelotii* rich in α-ESA and *Punica granatum* (pomegranate) rich in PunA. Therefore, the use of these seeds could be a potential dietary source of CLA, due to the conversion of α-ESA and PunA to RmA.

DHA is now considered as an essential fatty acid that should be provided by the diet at a minimum level of 250 mg/day. According to the last dietary survey performed in Belgium in 2014, that level of intake is far from being reached. Any attempt to increase the level in DHA in commonly consumed food items is thus worth being encouraged. In that respect, laying hens are precious partners since they very efficiently convert dietary ALA (provided by flaxseeds for example) into DHA and transfer it to the yolks of their eggs. As a result of the incorporation of sufficient ALA in hens' feed, the level of DHA can increase from about 20 mg/egg to 80-100 mg/egg.

The general purpose of this thesis was to improve the nutritional quality of egg yolk fat through the diet of laying hens. To achieve this objective, several experimental approaches have been implemented. More specifically, this study focused on three points:

1. the conversion of α-ESA and PunA after ingestion in the laying hen into RmA, and the transfer of these fatty acids into the lipids of the yolk

2. the changes in the lipids of the egg induced by the incorporation of α-ESA or PunA in hens' feeds enriched with flaxseeds
3. the effect of consuming enriched eggs as a functional food providing conjugated and omega-3 fatty acids in the human diet

The major outcomes derived from these items are presented in the next paragraphs.

The incorporation of α-ESA and PunA in the diet of the hens allowed a considerable increase in the RmA content in the egg yolk, while α-ESA and PunA were deposited differently in the lipids of the yolk

We investigated the metabolization of two CLnA isomers, α-ESA and PunA in hens. RmA has been identified as the primary CLA isomer in the lipids of eggs from hens fed with α-ESA or PunA. This is consistent with previous *in vitro* and *in vivo* studies hypothesizing the presence in animal cells of a Δ13-reductase activity, which would reduce the double bond in position 13 of PunA and α-ESA to form RmA (Schneider, Mignolet, Schneider, & Larondelle, 2013; Tsuzuki et al., 2006).

The experiments described in Chapters 5 and 6 have shown that the concentration of RmA in egg yolk lipids increased linearly as the dietary concentration of α-ESA or PunA increased. Interestingly, a maximum level of RmA in yolk lipids was reached 10 days after α-ESA or PunA were included in the diets tested in the short-term experiments described in Chapter 5, in which each hen was followed individually. The results corresponding to the long-term feeding trial presented in Chapter 6 confirm that the level of RmA increased rapidly but highlight that the maximum RmA level took more time to be reached when a population of hens is followed in a regular production unit. Indeed, Chapter 6 indicates that rearing conditions grouping together a large number of hens in the

same pen, and feeding with PSO over a long period, require more than one month to achieve stability in the concentration of RmA in eggs. In such a context, the individual features of the animal including the time to accept the feed and the metabolic efficiency influence the flock average. Individual monitoring of the hens during the experiments in Chapter 5 showed that certain hens rapidly reached a stable fatty acid profile in their eggs. Besides, certain hens had a high efficiency of converting α-ESA or PunA to RmA and/or transferring RmA into eggs, thus producing eggs with very high levels of RmA. Taken together, our results indicate that the time required for the RmA to reach a steady state accumulation in the egg yolk is influenced by several factors related to the animal or the diet, including the time required for the hen to adapt to the diet, its metabolic efficiency, the nature of the dietary fatty acids and the concentration of the CLnA precursor in the feed.

Apart from being a substrate for β-oxidation, RmA can be converted into several metabolites through desaturation and elongation processes. Thus, it forms a conjugated diene C18:3 (by the introduction of a double bond at position 6), a conjugated diene C20:3 (by elongation with two carbon atoms) and a conjugated diene C20:4 (by the introduction of a double bond at position 5) (Banni et al., 2001; Sébédio et al., 2001). In Figure 8.1, we suggest the same metabolic processes for α-ESA and PunA, which could lead to the formation of conjugated trienes C18:4, C20:4 and C20:5. Similar to RmA, we assume that some conjugated C16 could also derive from peroxisomal β-oxidation of α-ESA and PunA, as well as of their desaturated and elongated metabolites. However, the only metabolites found in egg yolks were cis 7, trans 9-hexadecadienoic acid and c6,c9,t11-CLnA derived from RmA. Thus, it appears that α-ESA and PunA are primarily converted to RmA, or stay unchanged before being transferred in the egg yolk, leading to an insufficient formation of secondary metabolites (conjugated C16 and C20) to allow a detectable transfer to eggs. The conversion rates for α-ESA and PunA were not calculated in our study. Therefore, their

quantities in egg yolks do not reflect their real conversion and/or transfer rates, but accumulated non-metabolized products. Both CLnA allowed considerable accumulation of RmA in egg yolks. By incorporating 7% (wt.) PSO into hens' feed, concentrations of more than 650 mg of RmA per egg could be reached, making these eggs effective contributors in the search for increased dietary CLA intakes. In that respect, a level of 3 g CLA/day has been proposed for adults to obtain potential health benefits. At the same time, significant proportions of PunA accumulated in the eggs. In contrast, the deposition of α-ESA was limited in the eggs of hens fed with *R. heudelotii* oil.

Our results confirmed previous findings (Schneider et al., 2013; Yuan, Sinclair, Zhou, & Li, 2009) that α-ESA is better converted than PunA into RmA. The only difference between these two isomers is the geometric structure of the double bond in position 13, which is *trans* in α-ESA, while it is *cis* in PunA. It is, therefore, possible that the enzyme involved in the saturation activity of the double bond in position 13 of α-ESA and PunA has a greater affinity for the *trans* configuration, hence the difference observed in the conversion efficiency of these fatty acids. Further studies are needed to provide a clearer understanding of the conversion mechanism of α-ESA and PunA into RmA.

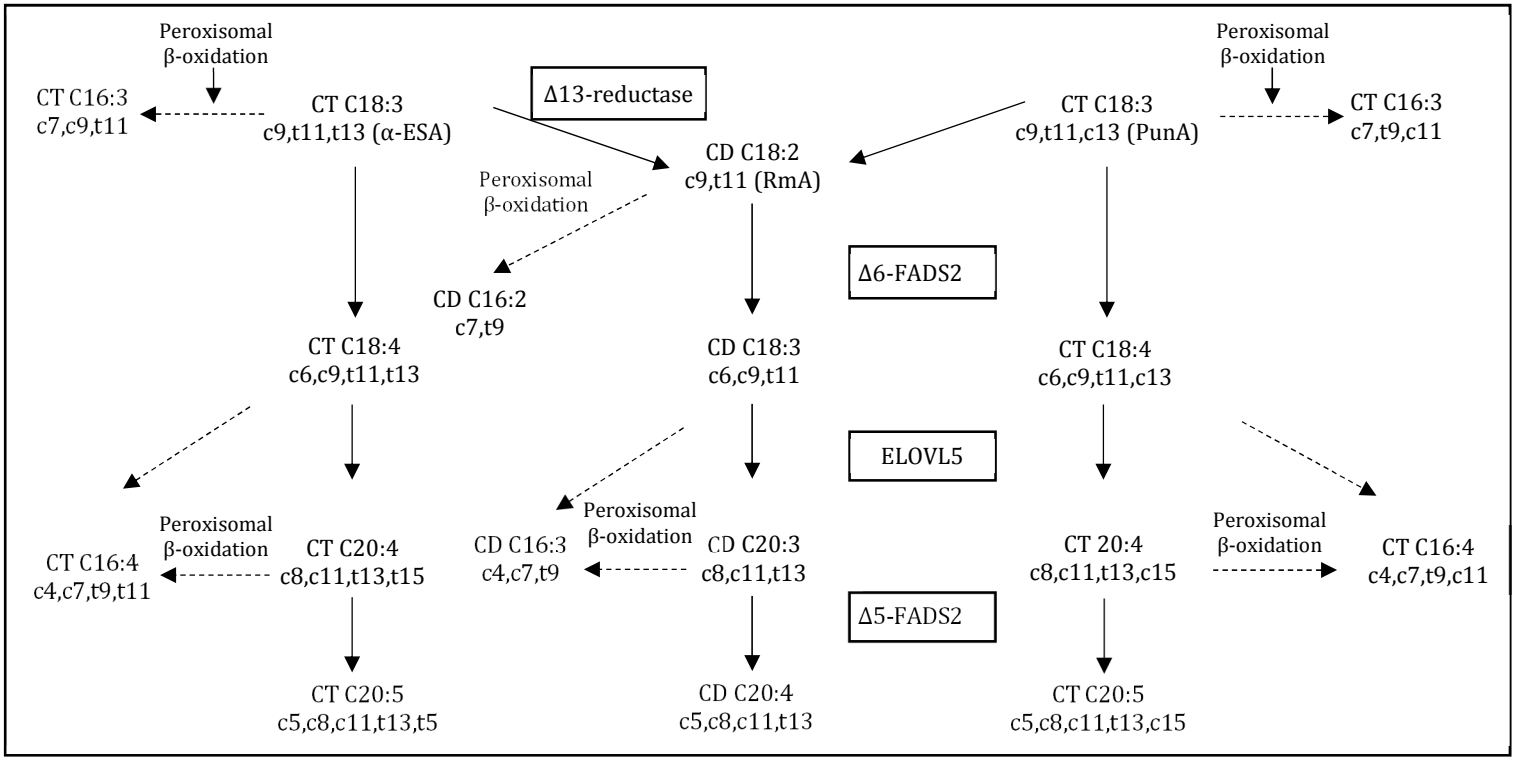

Figure 8.1. Metabolism of conjugated linolenic acids. Alpha-eleostearic acid (α-ESA) and punicic acid (PunA) are converted to rumenic acid (RmA) via a Δ13-reductase activity. A-ESA, PunA and RmA can undergo Δ6-desaturation, elongation and further Δ5 desaturation, while maintaining their conjugated triene (CT) or conjugated diene (CD) structure. Other metabolites with 16 carbon atoms, derived from peroxisomal β-oxidation of α-ESA, PunA and RmA can also been formed. Δ6 FADS2, Δ6 fatty acid desaturase 2; ELOVL5, very long fatty acid elongase 5; Δ5 FADS2, Δ5 fatty acid desaturase 2.

The incorporation of α-ESA and PunA into the feed of hens altered differently the accumulation of fatty acids in egg lipids

In the experiments of Chapter 5, we used feeds enriched with flaxseeds, which provided the hens with a source of omega-3 fatty acids. Eggs from hens fed with flaxseeds allowed the ALA content to be multiplied by 15 compared to a standard Belgian egg, while that of DHA (about 80 mg/egg) was multiplied by 4 (Table 5.4). In Chapter 6, the amount of n-3 PUFA, particularly the deposition of long chain n-3 PUFA through the inclusion of flaxseeds in the diet of hens, was significantly improved as compared to what was obtained with the flaxseed-free control.

The deposition of DHA in egg yolks was much higher than that of EPA, indicating that EPA is only an intermediate in the anabolic pathway converting ALA into DHA. Studies using fish oils composed of higher concentrations of EPA relative to DHA resulted also in greater enrichment of eggs with DHA than with EPA (Herber & Van Elswyk, 1996; Lawlor, Gaudette, Dickson, & House, 2010), highlighting once again the imperative need to introduce DHA in eggs to secure the optimal development of the chick.

In Chapter 5, feeding hens with diets containing a similar amount of ALA but increasing levels of α-ESA, resulted in increasing concentrations of ALA in eggs, while the DHA content showed a limited downward trend. The theory that CLA can modulate the activity of Δ6-desaturase has been widely mentioned to explain the decrease in the amount of ARA and DHA in the eggs of hens fed with CLA (Liu, Zhang, Yan, Shi, & Wei, 2017; Szymczyk & Pisulewski, 2003). The CLA could compete with LA and ALA for Δ6-desaturase. This suggests that fewer of these two acids were converted into their respective long-chain derivatives ARA and DHA, hence, the accumulation of LA and ALA and the decrease of ARA and DHA in eggs.

Two facts may explain the higher LA content in eggs from hens fed with α-ESA or PunA. First, the concentration of LA in the

diet, which was increased by the addition of oil from *R. heudelotii*. Second, the competition with RmA or possibly α-ESA or PunA for Δ6-desaturase, leading to the accumulation of LA in the eggs. Of note, the ARA content of yolks was not affected after the inclusion of α-ESA or PunA in ALA-rich diets (Chapter 5), which speaks against the second option. In contrast, we registered a significant decrease in the concentration of ARA in eggs from hens fed on flaxseeds and PSO, as compared to eggs from hens fed on a low ALA diet (Chapter 6). These results may indicate that the level of ALA in the diet of hens rather than α-ESA or PunA has played an important role in inhibiting the conversion of LA into ARA. As a whole, the increase in LA in the yolks was not a dietary concern in our case since the inclusion of flaxseeds allowed a greater increase in ALA, thus decreasing the n-6/n-3 ratio in the eggs. A significant increase in the egg content of SFA was observed with a concomitant reduction in MUFA, especially oleic acid, as reported by other poultry researchers who suggested a role of RmA or PunA in inhibiting Δ9-desaturase (Cherian, Gonzalez, Ryu, & Goeger, 2007; Kostogrys et al., 2017).

Incorporating α-ESA or PunA in hens' diets did not alter the total lipids or the total cholesterol content of yolk, because these components are regulated and kept within narrow ranges by the hen. The assumption that high levels of cholesterol and lipids in certain foods could play a major role in the onset of cardiovascular diseases through raising plasma cholesterol has aroused a reluctance in consumers towards the eggs (Herron & Fernandez, 2004). Several studies have sought to reduce the cholesterol content of the egg by acting on the hen through food, genetics or pharmacology. These attempts ended in failures or too limited effects. This near impossibility to decrease the cholesterol content of the egg is attributable to the fact that 95% of this molecule is associated with VLDL, of which 80% in a non-esterified form, associated with phospholipids on the surface of VLDL. Only the esterified part present in the nucleus, which represents only 20%

of total cholesterol, is potentially modifiable (Hermier, 1997; Nys & Sauveur, 2004). Conversely, and in line with our findings, it has been reported that a high-fat hen's diet can increase the cholesterol content of the egg (Nys & Sauveur, 2004).

Eggs rich in ALA, DHA, PunA, and RmA are functional foods whose regular consumption provides health benefits in humans

For considering the enriched eggs as a delivery system for functional fatty acids to humans, it is necessary to take the following considerations into account:

- Possible toxic effects of foods or nutrients for the laying hens

Published data on the toxic effects of flaxseeds on laying hens are inconsistent. After feeding hens on diets containing 0 or 10% flaxseeds for 25 weeks, Bean and Leeson (2003) reported a higher incidence of hepatic hemorrhage in hens fed flaxseeds compared to the control group. These results contradict those of Cherian and Hayat (2009), who reported no effect of long-term flaxseed feeding on the hepatic hemorrhage scores in hens. In addition, the inclusion of flaxseed in the hens' ration at levels greater than 10% has been reported to have a negative effect on egg production. The reduced production has been attributed to low nutrient utilization due to anti-nutritional factors present in flaxseed such as mucilage, cyanogenic glycosides or trypsin inhibitors (Beheshti Moghadam & Cherian, 2017). To minimize variations in egg quality and hens' health problems, it is common to use flaxseed inclusion rates of no more than 10% and to limit the feeding period to 30 to 60 weeks (Bean & Leeson, 2003; Cherian & Hayat, 2009).

The literature does not provide any research data on potential toxic factors of *R. heudelotii* kernels. However, the work of Tchiégang and colleagues suggests a high nutritional and health

potential of this seed (Mezajoug Kenfack, Arab–Tehrany, Tchiégang, & Linder, 2011; Tchankou Leudeu, Tchiégang, Barbé, Nicolas, & Guéant, 2009).

The toxicology and safety assessment of PSO in rats showed that dietary exposure to PSO at 13.7 g PSO/kg body weight per day for 28 days increased activity of liver enzymes (aspartate and alanine aminotransferases and alkaline phosphatase) and liver/body weight ratios. The no-observed-adverse-effect dose was 4.3 g PSO/kg body weight/day (Meerts et al., 2009).

In the framework of our study, the inclusion rates used for flaxseeds and oils from *R. heudelotii* or *P. granatum* had no adverse effects on both egg production and hens' health. For the particular case of the long-term feeding trial described in Chapter 6, we fed the hens approximately 4.7 g PSO/hen/day for 26 weeks and did not notice any adverse effects on the health of the hens. However, the incorporation of oils (RHO, PSO and olive oil) into the feed of hens led to a drastic increase in the fat content, which reached twice the recommended maximum value for hens. With the exception of calcium, which decreased slightly, the other nutrients were not affected and their content in the diets covered the nutritional needs of the hens. The calcium requirements of the hens were supplemented by a separate calcium feed (Chapter 6, Table 6.6). The main consequence of the high fat content of the feed was a decrease in the feed consumption of the hens. No fattening was observed. Another concern of the incorporation of CLnA-rich oils in the feed of hens is the lipid oxidation, which is likely to lead to a deterioration of the quality of the feed. In RHO, the content of α-tocopherol has been reported to range from 0.31 to 0.33 mg/g (Nzali, Tchiegang, Sandjon, & Meurens, 2016). The total tocopherol (α-, γ-, δ-tocopherols, plastochromanol 8 and γ-tocotrienol) contents of PSO can reach 3 mg/g, with α-tocopherol varying from 0.07 to 1.74 mg/g (Fernandes et al., 2015; Hajib et al., 2021; Pande & Akoh, 2009). However, α-ESA and PunA are highly sensitive to oxidation (Yang, Cao, Chen, & Chen, 2009). We managed the

stability of the oils by preparing the feeds daily or by storing them under vacuum in a cold room.

- Amount of nutrient delivered with an egg compared to the recommended daily allowance (RDA)

Analysis of the fatty acid composition of the tissues of laying hens receiving a diet enriched with flaxseeds and PSO showed that the priority fate of ALA, DHA, PunA and RmA is the yolk of the egg, compared to muscle. The eggs presented in Chapters 6 and 7 were enriched in ALA, DHA, PunA and RmA, so that the consumption of a single egg could cover respectively in adult men and women 4% and 5% of the RDA in ALA (2.8 and 2.2 g/day for men and women, respectively), along with 33% of the RDA in DHA (minimum 250 mg/day). Based on animal studies, a single egg would also respectively provide about 16% and 20% of the estimated doses of PunA (2 – 3 g/day) and RmA (3 g/day) required to elicit beneficial health effects in adults.

- Egg quality characteristics

Several studies reported changes in color and texture of yolk due to the incorporation of synthetic CLA in the hens' diet (Ahn, Sell, Jo, Chamruspollert, & Jeffrey, 1999; Cherian, Goeger, & Ahn, 2002). Deterioration of quality aspects during storage of CLA-enriched eggs has also been reported (Ahn et al., 1999; Chamruspollert & Sell, 1999).

The inclusion of PSO in the diet of hens has been shown not to alter the organoleptic properties of hard-boiled eggs, including odor, taste and appearance. In contrast, the color measurement showed that an increased concentration of PSO in the feed of the hens reduced the brightness (parameter L*), while increasing the parameter b* (negative values indicate blue and positive values indicate yellow) and the parameter a* (negative values indicate green and positive values indicate red) (Kostogrys et al., 2017). However, these analyses were carried out on eggs enriched with only PunA and RmA, after 12 weeks of feeding the hens on diets with a maximum inclusion rate of 2.5% of PSO. In the opinion of the

subjects involved in the experiment presented in Chapter 7, the quality of the enriched eggs with n-3 PUFA, PunA and RmA did not differ from those commonly marketed. Studies investigating the quality parameters of these eggs are however still needed.

The assessment of the stability of the fatty acid profile of the eggs during different culinary processes showed that the levels of ALA, DHA, RmA and PunA in the eggs have not been altered by cooking. Thus, these eggs can be easily integrated into a common diet as an important source of health-promoting fatty acids (Chapter 7, Figure 7.5).

- Established health-promoting properties

Although CLA enrichment of egg yolk has been well documented, studies reporting the health benefits of CLA-rich eggs are limited. Among the few studies found in the literature, the work of Franczyk-Zarów and colleagues (2008) showed that CLA-enriched eggs exerted an anti-inflammatory effect in mice. An *in vitro* study showed that the fatty acid extract from CLA-enriched egg yolks inhibited the growth and proliferation of the human breast adenocarcinoma cell line MCF-7 (Koronowicz et al., 2016). In both studies, a synthetic mixture of RmA and t10,c12 isomer was administered to hens to produce eggs. The trial described in Chapter 7 took up the challenge of studying for the first time in humans the health benefits associated with the consumption of eggs enriched in RmA, but also in PunA, ALA and DHA. The positive control OA-rich eggs intended to show that the association of n-3 PUFA with conjugated fatty acids is at least as effective as OA in the management of metabolic disorders. OA is one of the most abundant fatty acids found in the diet. Thus, its amount in the control eggs did not induce a significant difference in its dietary intake between the test and control groups. In addition, due to the low palmitic acid and LA content, the ratio of total unsaturated acids to total SFA in OA-rich eggs was of 2, which is similar to that of multi-enriched eggs.

The main health claim that may be deduced from the experiment performed on volunteers and described in Chapter 7 deals with the reduction of abdominal obesity. This positive effect is likely to be attributable to PunA and/or to some minor conjugated fatty acids identified in the eggs (Chapter 6). Indeed, RmA has not been reported to have an impact on adiposity, while ALA and DHA are provided at limited levels as compared to those that could be expected to have an anti-obesity impact. Interestingly, our results have shown that consuming approximately 650 mg PunA/day for three months has led to a significant impact on abdominal obesity in humans.

An undesirable effect observed during the study with the volunteers is that 50% of participants experienced an increase in cholesterolemia (HDL and LDL cholesterol), probably due to a high dietary cholesterol intake resulting from the egg consumption.

The dietary assessment performed on the volunteers revealed that for the average participants, the RDA in ALA and DHA were not met. This would likely prevent positive effects on some studied endpoints, including insulinemia, blood sugar and vascular health. This last consideration highlight the fact that the eggs enriched in ALA, DHA, PunA and RmA should be integrated into a healthy and balanced diet to offer the whole potential of their benefits.

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Chapter 9

General conclusion and future considerations

Dietary factors are considered major contributors to the development of non-communicable diseases, including cardiovascular diseases, type 2 diabetes and cancers. Epidemiological findings, supported by human and animal studies, have led to the recommendations that people should consume at least two servings of fruits and three servings of vegetable daily, reduce SFA intake and eat at least two servings of fish weekly. However, as the latest Belgian dietary survey revealed, people fall short of meeting healthy eating guidelines (De Ridder et al., 2016). In line with this observation, the recommended nutritional intakes are not covered for several essential nutrients. Thus, it seems likely that the production of functional foods enriched with various nutrients could be a way to compensate for their lack in our general diet. This work aimed to improve the nutritional quality of eggs by increasing their content in n-3 PUFA (ALA and DHA), conjugated linolenic acids (α-ESA or PunA) and conjugated linoleic acid (RmA). The strategy adopted was to add in the hens' diets flaxseeds and non-traditional seed oils namely, *R. heudelotii* kernel oil and *P. granatum* seed oil. In this thesis, we demonstrated the followings:

It is possible to provide consumers with a range of animal-derived products with improved nutritional composition in such a way that they can deliver a substantial amount of health-promoting nutrients. Supplementing the diet of hens with flaxseeds combined with a source of CLnA improves the fatty acid profile of the eggs and therefore their nutritional quality. Enriched eggs are intended to help improve the diet by providing a significant amount of essential nutrients without changing dietary preferences. In addition, due to their culinary versatility, such eggs could be used as an alternative to fish (for DHA intake) or ruminant-derived foods (for RmA intake) to vary meals and avoid the monotony that characterizes most healthy diets.

1. The bioconversion of α-ESA and PunA to RmA is effective in hens, which transferred substantial amounts of RmA and PunA to the yolk

Our hypothesis that foods rich in CLnA are an indirect source of CLA for animals was confirmed. In laying hens, egg yolk has priority over tissues, for the transfer of α-ESA, PunA and RmA, as is the case for other dietary fatty acids. This led to a significant accumulation of PunA and RmA in egg yolk, as well as metabolic derivatives of RmA. The amount of α-ESA and PunA used in this research did not allow detecting fatty acids derived from α-ESA and PunA other than RmA in eggs. Additional studies on a variety of animal models including rodents and fishes should be considered to further investigate and compare the metabolic pathways of CLnA in different animals.

2. Dietary α-ESA and PunA have no negative effect on the capacity of the hen in bioconverting ALA into DHA

Because of their close structures, α-ESA and PunA are likely to compete with ALA for long-chain PUFA bioconversion. The absence of desaturated and elongated α-ESA and PunA metabolites in eggs indicates that ALA is the preferred substrate for the enzymes of this metabolic pathway used for the formation of DHA.

3. The nutritional strategy developed is safe and can be deployed in a farm or an egg production unit

The egg enrichment strategy was implemented for a period of 6 months with no adverse effects observed on the hens. However, for a broad extension of such a feeding program, several factors remain to be taken into account, including the nutritional balance of rations given to hens, the availability and cost of feed ingredients and the organoleptic characteristics of the eggs produced. The supplementation of hens' feeds with CLnA-rich oils

involves two major problems, namely the risk of fattening hens and lipid oxidation of feeds due to their high PUFA content. A supplement of protein in the diets may be considered in order to balance high amounts of fat. To prevent lipid peroxidation, antioxidants (polyphenols such as those of green tea extracts, vitamin E, carotenoids, etc.) can be used. They would also ensure the protection of the PUFA at the level of the eggs to which they would be transferred (Fisinin, Papazyan, & Surai, 2009; Grobas, Méndez, Lopez Bote, De Blas, & Mateos, 2002; Ren, Wu, & Renema, 2010). The eggs we developed are intended to be a dietary carrier for essential or health-promoting fatty acids. Sensory characteristics are an important factor in consumer acceptance. Therefore, studies on the physical, chemical and functional properties of these eggs are needed. Another issue deals with the additional cost of the animal feed resulting from the addition of oils, which should lead to an increase in the price of eggs for the consumers. In order to reduce production costs, dedicated production and supply lines for *R. heudelotii* or *P. granatum* oils should be set up.

4. The consumption of eggs rich in ALA, DHA, PunA and RmA acids reduces abdominal obesity.

The clinical trial presented in Chapter 7 has shown the absence of deleterious effects of the consumption of the enriched eggs, with the exception of a very limited but significant increase in ox-LDL. Among the parameters that were followed, the waist circumference showed a significant decrease in the test subjects, suggesting an impact on abdominal fat. Further investigations with a larger number of volunteers are needed to confirm this observation. The way the eggs are consumed should also be evaluated. Indeed, eggs are eaten as such or used in the wide range of culinary preparations. The question of the health effects of dietary products derived from the enriched eggs developed in the present thesis should thus be addressed.

The underlying mechanisms leading to a reduction of abdominal obesity as a result of the consumption of the ALA-DHA-RmA-PunA enriched eggs still need to be elucidated. Among others, clinical investigations deciphering the modifications of the adipose tissue content and their metabolism should be put in place. In that respect, a human trial using dietary crackers derived from the egg yolks and following the abdominal tissue composition and liver adiposity through the use of scanners is on the way. In addition to this *in vivo* trial, subcutaneous adipocytes may be isolated from the abdominal region in obese or nonobese subjects and incubated with fatty acids individually or in combination. The lipolytic effect of fatty acids could be assessed by measuring the levels of glycerol released in the different incubation media. To elucidate the mechanisms behind lipolysis, the status of adrenoreceptors involved in the lipolytic cascade including the activation of the protein kinase/hormone-sensitive lipase complex may be assessed. Experiments could also be set up on animal models fed on the test or control eggs, as well as on small animals, such as zebrafish, that would be fed on experimental diets enriched in one specific fatty acid or in defined combinations thereof. A whole set of parameters could then be followed, including the level of expression of genes associated with lipid metabolism in different key organs, and the activity of the resulting proteins. Regarding the oxidative stress observed in Chapter 7, feeding trials in animal models, which would be provided with different combinations of ALA, DHA, RmA and PunA in a high-fat diet compared to a normal diet, would make it possible to know if one or several of the fatty acids under investigation are responsible for the registered increase in ox-LDL.

Finally, since the literature indicates that α-ESA and PunA can have diverging biological effects (Koba, Belury, & Sugano, 2007), animal and human trials should be set up to compare the eggs produced with the addition of PSO in hens' diet with eggs obtained by incorporating α-ESA into the hens' feed. As shown in

Chapter 5, these eggs contained low amounts of α-ESA associated with high levels of ALA, DHA and RmA, allowing thus to explore the health effect of eggs enriched in omega-3 (ALA, DHA) and omega-7 (RmA) fatty acids but with a very low level of omega-5 CLnA. This would allow highlighting potential differences in the effects induced by the eggs produced from the two different CLnA under investigation, and would confirm or infirm the central role of CLnA and more specifically of PunA in the reduction in waist circumference observed in Chapter 7.

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