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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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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.

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 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 (Channugam 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

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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 (Shinohara et al., 2012; Arao et al., 2004). 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.

2. Materials and methods

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.

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.

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 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.

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.

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 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).

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, 2 M), with incubation in a shaking water bath at 50 °C for 2 h. 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.

Table 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.

The analysis of the cholesterol trimethylsilyl (TMS) derivative was performed with a GC equipped with an RXi-5Sil MS column (30 m × 0.25 mm internal diameter × 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 1 μl 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).

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 $p \leq 0.05$.

3. Results

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 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 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 1).

Diet composition had a significant effect on the hen's feed consumption (Table 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).

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 Figs. 1 and 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 (Fig. 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 (Fig. 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: $p = 0.9210$) (Fig. 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 less than 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 (Fig. 2**.B**).

3.3. Fatty acid composition of egg yolk

On the basis of the observations in Fig. 2, the fatty acid compositions of yolks (in mg/g of yolk) presented in Table 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

Table 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 (mean value ± standard deviation).

	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 in the same row within a trial with no common superscript letter 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.

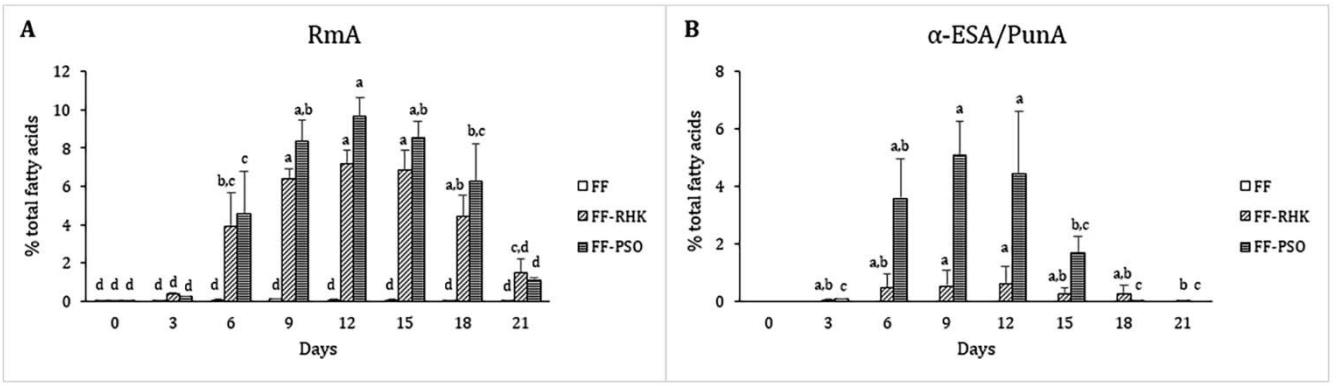

Fig. 1. Evolution of the proportions (wt.%) of rumenic acid (RmA) (**A**), and α-eleostearic acid (α-ESA) or punicic acid (PunA) (**B**) in the egg yolks of hens fed for 12 days with diets supplemented with kernels of *R. heudelotii* or pomegranate seed oil, and then returned to the control diet (days 12 to 21). Values are means ± standard deviations. Mean values within the same diet with no common letter 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.

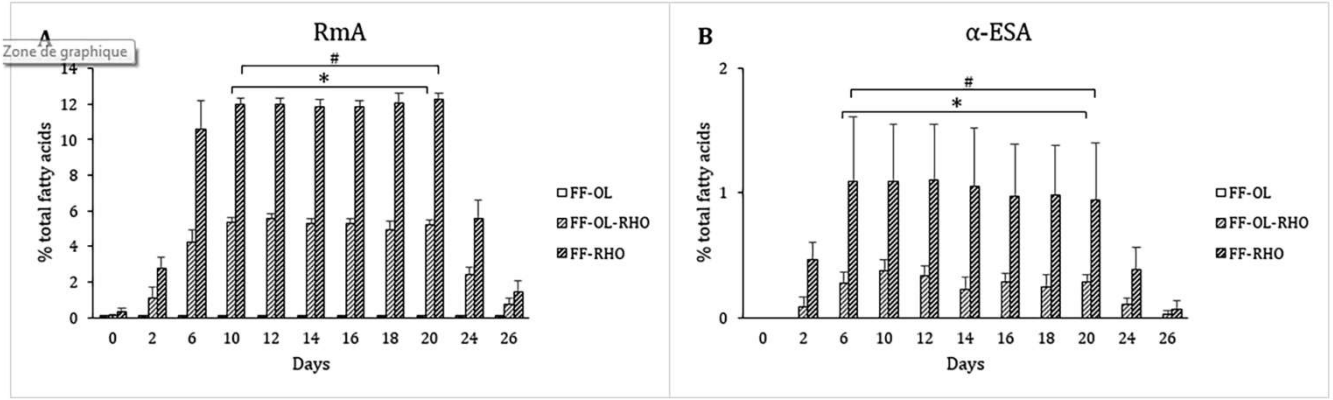

Fig. 2. Evolution of the proportions (wt.%) of rumenic acid (RmA) (**A**) and α-eleostearic acid (α-ESA) (**B**) in the egg yolks of hens fed for 21 days with diets supplemented with *R. heudelotii* oil, and then returned to the control diet (days 21 to 26). Values are means ± standard deviations. (*) 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.

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 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.

4. Discussion

Feeding synthetic CLA oil has been the most widely used and

Table 3
Effect of diets on fatty acid composition of egg yolk (mg/g of yolk) (mean value ± standard deviation).

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)	$0.00^{a} \pm 0.00$	$1.36^{a} \pm 0.39$	$13.70^{b} \pm 2.86$	$0.00^{a} \pm 0.00$	$0.88^{b} \pm 0.26$	$3.01^{c} \pm 1.14$
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)	$5.34^{a} \pm 0.70$	$5.01^{a} \pm 0.72$	$5.86^{a} \pm 0.53$	$5.55^{a} \pm 0.77$	$5.22^{a} \pm 0.68$	$4.95^{a} \pm 0.55$
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 in the same row within a trial with no common superscript letter 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. SFA: Saturated fatty acids. MUFA: Monounsaturated fatty acids. n-6 PUFA: Omega-6 polyunsaturated fatty acids. n-3 PUFA: Omega-3 polyunsaturated fatty acids.

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 and colleagues and Schneider and colleagues 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 (Yuan, Sinclair, Zhou, & Li, 2009c; Schneider et al., 2013). 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 (Shinohara et al., 2012; Arao et al., 2004). 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. Implications

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 egg composition and hens' health. 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.

CRediT authorship contribution statement

M.T. Ngo Njembe: Conceptualization, Methodology, Investigation, Data curation, Validation, Visualization, Writing - original draft. **E. Dormal:** Investigation, Data curation. **C. Gardin:** Investigation, Resources, Data curation. **E. Mignolet:** Software, Resources. **C. Debier:** Writing - review & editing. **Y. Larondelle:** Conceptualization, Visualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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