Impact of different omega-3 polyunsaturated fatty acid (n-3 PUFA) sources (flaxseed, *Isochrysis galbana*, fish oil and DHA Gold) on n-3 LC-PUFA enrichment (efficiency) in the egg yolk.

Charlotte Lemahieu[a,1,*], Charlotte Bruneel[a,1], Eline Ryckebosch[a,1], Koenraad Muylaert[b], Johan Buyse[c,1], and Imogen Foubert[a,1]

[a]Research unit Food and Lipids, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium.

* Corresponding author: Charlotte Lemahieu

Phone: +32 (0) 56 24 61 72

Fax: +32 (0) 56 24 69 99

E-mail: charlotte.lemahieu@kuleuven-kulak.be

[b]Research unit Aquatic Biology, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium.

[c]Division of Livestock-Nutrition-Quality, KU Leuven, Kasteelpark Arenberg 30, 3001 Leuven, Belgium.

[1]Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium

ABSTRACT

Four different omega-3 polyunsaturated fatty acid (n-3 PUFA) sources (flaxseed, *Isochrysis galbana*, fish oil and DHA Gold) were supplemented to the diet of laying hens in such a way that the same amount of extra n-3 PUFA (120 mg per 100 g feed) was added to the diet and enrichment of egg yolk with n-3 PUFA was monitored. The obtained n-3 long chain (LC)-PUFA enrichment was not as efficient for all n-3 PUFA sources. The lowest enrichment efficiency (≈6%) was observed when flaxseed (α-linolenic acid source) was supplemented. Drastically higher n-3 LC-PUFA enrichment efficiencies were observed with supplementation of the n-3 LC-PUFA sources. However, for the n-3 LC-PUFA sources (fish oil, *Isochrysis galbana* and DHA Gold) differences in enrichment efficiencies were observed (≈55%, ≈30% and ≈45%, respectively), this because of different bio-accessibility of the n-3 PUFA and different n-3 PUFA profiles of the three sources.

KEYWORDS

n-3 LC-PUFA enrichment

Flaxseed

Fish oil

Isochrysis galbana

DHA Gold

Egg yolk

1. Introduction

Since the last decades, there is a growing interest to enrich food products with omega-3 polyunsaturated fatty acids (n-3 PUFA). Several health benefits, like the reduction of cardiovascular diseases and the development of visual and cognitive functions in foetus and young children, are associated with n-3 PUFA. Long chain (LC) n-3 PUFA, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), appear to have a much stronger effect than the shorter chain n-3 PUFA α-linolenic acid (ALA) (Bourre, 2005; Gogus and Smith, 2010; Harris, 2007; Jeyakumar, 2012; Jordan, 2010; Simopoulos, 1999; Trautwein, 2001; Yashodhara *et al*., 2009). The conversion of ALA to EPA and DHA in the human body is rather limited (Trautwein, 2001; Burdge, 2004; Komprda, 2012). Despite the proven health benefits, the intake of these n-3 PUFA, and more particularly n-3 LC-PUFA, does not meet the recommended intake of 250 mg per day in most countries worldwide (Burdge, 2004; Kris-Etherton *et al*., 2009; Meyer, 2011; Ruxton *et al*., 2004; Sioen *et al*., 2006; Sioen *et al*., 2009). In this respect, enrichment of food products with n-3 LC-PUFA has received an increasing interest in the past few years (Fraeye et al., 2012; Morato et al., 2015; Stratulat et al., 2015).

Eggs are a very good food product to enrich with n-3 LC-PUFA. First of all, they are consumed by most people worldwide, as such or processed in other foodstuffs. Secondly, the fatty acid profile of the egg yolk, where the lipids are concentrated, is closely linked to the type of lipids consumed by the laying hens (Bourre, 2005; Fraeye *et al*., 2012). To raise the level of n-3 PUFA in the egg yolk, several n-3 PUFA sources can be supplemented to the diet of the laying hens: flaxseed, fish oil, DHA Gold (heterotrophic microalgae) or autotrophic microalgae (Fraeye *et al*., 2012). The supplementation of the ALA source flaxseed has already been studied intensively (Fraeye *et al*., 2012). Flaxseed enriched feed mainly results in ALA enrichment, up

62 to 200 mg per egg, but also substantial increases of the DHA content, up to 90 mg per egg,
63 this with overdosing the amount of flaxseed (Aymond and Van Elswyk, 1995; Bean and Leeson;
64 2003; Fraeye *et al*., 2012). However, it is more interesting to enrich eggs with n-3 LC-PUFA,
65 supplementation with a direct source of EPA and/or DHA. Fish oil, as source of EPA and/or
66 DHA, for example, can be supplemented to the diet of the laying hens and mostly results in
67 DHA enrichment in the egg yolk (up to ± 100 mg per egg), while the EPA content increases to
68 a much lesser extent (Bovet *et al*., 2007; Cachaldora *et al*., 2008; Carrillo *et al*., 2008; Fraeye
69 *et al*., 2012; Gonzalez-Esquerra and Leeson, 2000; Van Elswyk *et al*., 1995). The
70 supplementation dose of fish oil, however, needs to be restricted, since inclusion levels of fish
71 oil above 1.5% in the diet of laying hens leads to eggs which are no longer acceptable for
72 human consumption, especially in Western countries (Gonzalez-Esquerra and Leeson, 2000;
73 Van Elswyk, 1997) because they are described as 'fishy' eggs by sensory panels (Van Elswyk,
74 1997). Even when deodorized fish oil or microencapsulated fish oil is used, a negative impact
75 on the sensory parameters of the egg is still observed (Gonzalez-Esquerra and Leeson, 2000;
76 Lawlor *et al*., 2010). It has been suggested that an even higher enrichment of n-3 LC-PUFA can
77 be obtained by the supplementation of heterotrophic microalgae, sold as e.g. DHA Gold, as
78 source of DHA (Fraeye *et al*., 2012). Herber and Van Elswyk (1996), for example, observed a
79 similar enrichment (± 130 mg n-3 PUFA per egg) by supplementation of 1.5% fish oil and 2.4%
80 DHA Gold. However, DHA levels up to 200 mg per egg can be obtained by supplementation of
81 these heterotrophic microalgae at higher doses (4.8%), while still obtaining eggs with an
82 acceptable taste (Herber-McNeill and Van Elswyk, 1998).

83 Limited research has also been performed to investigate the effect of the supplementation of
84 autotrophic microalgal species, as source of ALA or EPA and/or DHA, to the diet of the laying

hens. Supplementation of hens' feed with autotrophic microalgae provides the most environmentally sustainable approach to raise the n-3 LC-PUFA content in eggs. Autotrophic microalgae are the primary producers of these fatty acids. They only need light and CO_2 to produce their biomass, which is in contrast to heterotrophic microalgae which have to be fed organic molecules (Nuño et al., 2013). Depending on the autotrophic microalgal species used as feed supplement, different levels of n-3 LC-PUFA enrichment in the eggs can be obtained (Bruneel *et al*., 2013; Fredrikssen *et al.,* 2006; Lemahieu *et al*., 2013a; Nitsan *et al*., 1999).

There is a lack of studies that directly compare the effectiveness of different n-3 PUFA sources for enrichment of egg yolk with n-3 LC-PUFA. Moreover, the few comparative studies that are available do not supplement equal n-3 PUFA dosages. In this study, the four different n-3 PUFA sources: flaxseed, fish oil, DHA Gold and the autotrophic microalgal species *Isochrysis galbana*, were therefore supplemented to the diet of laying hens in one experimental set-up, which has, to the best of our knowledge, never been done before. Flaxseed, fish oil and DHA Gold are three n-3 PUFA sources which are commercially available and already used to enrich eggs with n-3 PUFA. In addition, based on the study of Lemahieu et al. (2013a), *Isochrysis galbana* was the most appropriate autotrophic microalgal species to enrich eggs with n-3 PUFA, so this microalgal species was included in this study. Moreover, the n-3 PUFA sources were supplemented in such a way that the same n-3 PUFA amount was added to the diet of the laying hens. This is important as earlier research showed that the supplemented n-3 PUFA amount has a drastic impact on the enrichment efficiency obtained in the egg yolk (Caston and Leeson 1990; Herber and Van Elswyk, 1996; Lemahieu *et al*., 2013a; Lemahieu *et al*., 2014; Van Elswyk, 1997;).

2. Materials and methods

2.1. N-3 PUFA sources

Four different n-3 PUFA sources were used in this study as a feed supplement: extruded flaxseed (AVEVE, Leuven, Belgium), *Isochrysis galbana* (Archimede Ricerche, Camporosso, Italy), fish oil (Inve België, Baasrode, Belgium) and DHA Gold (Bivit, Wevelgem, Belgium).

To determine the n-3 PUFA content of the different sources, the lipid fraction of the extruded flaxseed, *Isochrysis galbana* and DHA Gold was first extracted with chloroform:methanol (1:1, v:v) according to the method described by Ryckebosch *et al*. (2012). At the start of the extraction, an internal standard (C12:0) was added to calculate the amount of n-3 PUFA in the different sources. The fish oil was used as such, only the internal standard (C12:0) was added for quantification. The lipid fraction was then methylated according to Ryckebosch *et al.* (2012) and the n-3 PUFA profile was determined by gas chromatography as described by Lemahieu *et al*. (2013a). The results are shown in **Table 1** and discussed in section **3.1**.

2.2. Animals and diets

Forty ISA Brown laying hens (28 weeks of age, ‘t Munckenei, Wingene, Belgium) were housed in battery cages, two hens per cage, in an environmentally controlled room. The room temperature was set at 20 °C and the hens received 16 h of light per day. Feed and water were supplied *ad libitum*.

During the adaptation period of 14 days, the laying hens only received the commercially available standard diet (Legmeel Total 277, AVEVE, Wilsele, Belgium), in order to adapt to the new environment and the new diet. After these 14 days of adaptation, the 40 laying hens were

divided into five groups of eight hens. One of these groups, the control group, continued to receive only the standard diet, further referred to as control diet. The other four groups received the control diet supplemented with one of the four n-3 PUFA sources: extruded flaxseed, *Isochrysis galbana,* fish oil or DHA Gold. Earlier research by Lemahieu *et al*. (2014) showed that supplementation of 120 mg n-3 PUFA per 100 g feed, by addition of *Isochrysis galbana*, was the most optimal supplementation dose to reach the highest n-3 LC-PUFA enrichment efficiency. To make a correct comparison with the other sources, they were also supplemented to reach 120 mg extra n-3 PUFA per 100 g feed. Based on the fatty acid profile of the n-3 PUFA sources, obtained as described in **2.1** and shown in **Table 1**, the supplementation doses of the four n-3 PUFA sources were calculated: 0.56% extruded flaxseed, 2.03% *Isochrysis galbana*, 0.68% fish oil (which is below the maximum advised level of 1.5%) and 0.44% DHA Gold (all doses expressed on feed basis).

The supplementation of the different n-3 PUFA sources lasted 21 days (supplementation period). During this period, the average daily feed intake, the egg production and egg weight, and the mortality and the morbidity of the hens were registered on a daily basis.

2.3. Egg collection, storage and analysis

The eggs collected during the supplementation period were stored at -20 °C, until further analysis. The fatty acid profile of the eggs at the start and at the end of the supplementation period were determined according to the method described in Lemahieu *et al*. (2013a). Briefly, the lipid fraction of the egg yolk was extracted, after addition of an internal standard, with chloroform:methanol (2:1, v/v). The extraction was performed twice, and the combined chloroform:methanol extracts were washed with KCl (0.88%). Afterwards, the

150 chloroform:methanol was removed by rotary evaporation. The lipid fraction was then
151 methylated according to Ryckebosch *et al.* (2012) and the n-3 PUFA profile was determined
152 by gas chromatography as described by Lemahieu *et al*. (2013a).

153 **2.4. Statistical analysis**

154 The results were statistically evaluated by one way analysis of variance (ANOVA) and post-hoc
155 Tukey's test with α=0.05 (Sigmaplot 11, Systat Software Inc., Chicago, IL, USA).

3. Results and discussion

3.1. n-3 PUFA composition of the four sources

The n-3 PUFA content and profile of extruded flaxseed, *Isochrysis galbana*, fish oil and DHA Gold is shown in **Table 1**. Extruded flaxseed was source of the shorter chain n-3 PUFA ALA (21.4 ± 0.7 %). *Isochrysis* was mainly source of stearidonic acid (SDA) (2.67 ± 0.07 %) and DHA (1.78 ± 0.04 %), but also contained significant amounts of ALA (1.36 ± 0.03 %). Fish oil, on the other hand, was mainly source of EPA (7.06 ± 0.15 %) and DHA (6.76 ± 0.16 %), and contained much lower amounts of ALA (1.105 ± 0.003 %) and SDA (1.35 ± 0.03 %). DHA Gold was source of DHA (26.4 ± 0.3 %) and contained only very small amounts of the other n-3 PUFA.

3.2. n-3 (LC-) PUFA enrichment in the egg yolk

The supplementation of the different n-3 PUFA sources resulted in different enrichment patterns in the egg yolk (**Table 2**).

Feed supplementation with the ALA source flaxseed (120 mg extra n-3 PUFA per 100 g feed or 0.56 g flaxseed per 100 g feed) resulted in significantly higher amounts of ALA (19.2 ± 2.5 mg per egg) and DHA (33 ± 4 mg per egg) in the egg yolk in comparison with the ALA (9.9 ± 1.0 mg per egg) and DHA (21 ± 3 mg per egg) content in the eggs from the control group. This is in accordance with the results in the literature, although the absolute amounts cannot be compared as a different dose was supplemented (Caston and Leeson, 1990; Fraeye *et al*., 2012; Schiedeler and Froning, 1996; Van Elswyk, 1997). For example, diet supplemented with 15% of ground flaxseed increased ALA from 13 to 212 mg per egg and DHA from 28 to 90 mg per egg (Aymond and Van Elswyk, 1995). Based on the literature and the results obtained in this study, it can be concluded that ALA can be converted to DHA by the laying hens, but that

this is a rather inefficient process since also significant amounts of ALA were incorporated into the egg yolk, especially with very high supplementation doses of flaxseed (Aymond and Van Elswyk, 1995; Bean and Leeson, 2003). A second indication for the conversion of ALA to DHA by the laying hen is the slightly, but significantly, increased, docosapentaenoic acid (DPA) content (5.5 ± 3.0 mg per egg compared to 3.0 ± 0.5 mg per egg in the control group), as intermediate between ALA and DHA.

The three n-3 LC-PUFA sources (*Isochrysis galbana*, fish oil and DHA Gold) mainly resulted in DHA enrichment in the egg yolk, regardless of whether EPA or DHA was supplemented to the diet.

A DHA content of 92 ± 3 mg per egg was observed for the supplementation with fish oil (0.68 g fish oil per 100 g feed or 120 mg extra n-3 PUFA per 100 g feed). However, also a slightly, but significantly, higher EPA (3.5 ± 0.3 mg per egg) and DPA (10.0 ± 1.1 mg per egg) content was observed in comparison with the control group. This is, in general, in accordance with the results obtained by Cachaldora *et al*. (2008); Herber and Van Elswyk (1996) and Lawlor *et al*. (2010) although again exact comparison of enrichment amounts is not possible. It can thus be concluded that, with supplementation of fish oil, a conversion by the laying hens of EPA to DHA has occurred, since mainly DHA was observed in the egg yolk and an increase of the conversion product DPA was also obtained.

The DHA content obtained by the supplementation of DHA Gold (0.44 g DHA Gold per 100 g feed or 120 mg extra n-3 PUFA per 100 g feed) was very similar (not significant different) to the DHA content obtained by supplementation of fish oil (90 ± 5 mg DHA per egg compared to 92 ± 3 mg DHA per egg, respectively). No drastic changes were however observed for the

other n-3 PUFA, which is in accordance with literature and can be explained by the fact that no conversion processes are needed. DHA can be incorporated in egg yolk in a direct way (Cachaldora *et al*., 2005; Cachaldora *et al*, 2008; Cheng *et al*., 2004; Fraeye *et al*., 2012; Herber and Van Elswyk; 1996).

Also mainly DHA enrichment (67 ± 3 mg DHA in the egg) was observed in the egg yolk with supplementation of *Isochrysis galbana* (2.03 g per 100 g feed or 120 mg extra n-3 PUFA per 100 g feed), despite the high amounts of ALA and SDA supplemented (**Table 1)**. This confirmed once again that preferably DHA was deposited in the egg yolk. So, next to the direct incorporation of the supplemented DHA also conversion of ALA and SDA occurred.

3.3. n-3 PUFA egg enrichment efficiencies by supplementation of different n-3 PUFA sources

To compare the different sources, it is especially interesting to calculate the efficiency of the n-3 (LC-) PUFA enrichment in eggs. This is calculated as described in Lemahieu *et al*. (2013a) and the results are presented in **Table 3**.

The highest n-3 LC-PUFA enrichment, accompanied by the highest n-3 LC-PUFA incorporation efficiency, was obtained with the supplementation of fish oil (enrichment efficiency of ≈ 55%). A 10% lower enrichment efficiency (≈ 45%) and significantly different with the enrichment efficiency obtained by fish oil, was obtained with the supplementation of DHA Gold. This is in contrast to the results obtained by Herber and Van Elswyk (1996) who supplemented 1.5% fish oil and 2.4% heterotrophic microalgae, which led to approximately the same n-3 LC-PUFA enrichment in the egg yolk. However, much more n-3 PUFA were added by the supplementation of 1.5% fish oil in comparison with the supplementation of 2.4% microalgae.

This suggests a higher enrichment efficiency of supplementation with heterotrophic microalgae in comparison with fish oil, although the results are biased by a possible dose effect on the enrichment efficiency since the n-3 PUFA dose supplemented was not the same for both sources.

Supplementation of *Isochrysis* to the diet of the laying hens led to an enrichment efficiency of approximately 30%, which is significantly lower in comparison with fish oil and DHA Gold. But, this is also drastically lower in comparison with the research of Lemahieu *et al*. (2014) where the same microalgae was supplemented in the same n-3 PUFA dose (equivalent to 2.4% microalgal biomass) and an efficiency of 53% was observed. A possible explanation could be the percentage of DHA supplemented, which was somewhat (5%) lower in this study, since more SDA was present in the microalgal biomass. Different batches could also have a different digestibility, which could lead to different enrichment efficiencies. Next to this, also variation between different experiments can occur, which makes it important to compare the four different sources in the same study.

The lowest enrichment efficiency (≈ 6%) was observed with the supplementation of flaxseed. It is difficult to compare this efficiency with results obtained in literature since almost in all cases no information was given about the supplemented n-3 PUFA amount and as known, the supplemented n-3 PUFA dose has a significant influence of the enrichment efficiency.

Several suggestions can be made to explain the differences in enrichment efficiencies with the supplementation of the different n-3 PUFA sources. Based on the fatty acid profile of the n-3 PUFA sources, it could be expected that DHA Gold would lead to the highest enrichment efficiency since it is a direct source of DHA, which is the fatty acid preferentially stored in the

egg yolk. Cachaldora *et al*. (2006) also showed that supplementation of fish oil with different ratios of EPA/DHA leads to different incorporation efficiencies, with the highest efficiency obtained with the fish oil with the lowest EPA/DHA ratio, so, with the highest DHA content. However, in this study a higher n-3 LC-PUFA enrichment efficiency by the supplementation of fish oil was obtained. This can presumably be explained by the higher bio-accessibility of the n-3 PUFA, since these fatty acids were supplemented as oil. Based on the results obtained in this study, the bio-accessibility thus seems to have a greater impact on the enrichment efficiency than the type of n-3 LC-PUFA provided. However, to definitely conclude this, the n-3 LC-PUFA enrichment obtained by the supplementation of fish oil should be compared to the enrichment obtained by the supplementation of the oil of DHA Gold.

The n-3 LC-PUFA enrichment and the n-3 LC-PUFA incorporation efficiency obtained by supplementation of *Isochrysis galbana* to the diet of the laying hens was drastically lower in comparison with the supplementation of fish oil and DHA Gold. First of all, *Isochrysis* contained much higher amounts of ALA and SDA and a lower amount of DHA in comparison with fish oil and DHA Gold. This means that the percentage of DHA in the total supplemented n-3 PUFA was much lower for *Isochrysis galbana* and thus more conversion reactions are needed to raise the level of DHA in the egg yolk, which could decrease the efficiency of enrichment. Cachaldora *et al*. (2008) supplemented diets, rich in ALA and EPA/DHA, to laying hens by supplementation of flaxseed and fish oil and concluded that an excess of the n-3 LC-PUFA limits the conversion of ALA. The relative portions of the supplemented n-3 PUFA thus plays a crucial role in the n-3 LC-PUFA enrichment efficiency. Secondly, compared to fish oil, the lower efficiency could also partly be explained by the bio-accessibility. *Isochrysis* consist of a cell membrane/cell wall which could reduce the bio-accessibility in contrast to fish oil, where the

267 oil was supplemented as such (Wootton *et al*., 2007; Zhu and Lee, 1997). This could also be an
268 explanation for the lower efficiency compared to DHA Gold. Different microalgae consist of
269 different cell wall compositions which could affect the digestibility and lead to different
270 enrichment efficiencies (Lemahieu *et al.,*2013a). This parameter, bio-accessibility, should, by
271 the way, also be taken into account in the study of Cachaldora *et al*. (2008), since flaxseed and
272 fish oil probably also lead to a different bio-accessibility of the n-3 PUFA.

273 The lowest n-3 LC-PUFA enrichment (efficiency) was obtained with the supplementation of
274 extruded flaxseed to the diet of the laying hens. This could be expected since the conversion
275 of ALA to the n-3 LC-PUFA is a rather limited process (Aymond and Van Elswyk, 1995). Since
276 mostly ALA enrichment was observed in the egg yolk with supplementation of flaxseed (Fraeye
277 *et al*., 2012), it is interesting to also evaluate the total n-3 PUFA incorporation efficiency, next
278 to the n-3 LC-PUFA enrichment efficiency. However, the n-3 PUFA incorporation efficiency,
279 which includes the ALA enrichment in the yolk, was only slightly higher (10 ± 8 %) than the n-
280 3 LC-PUFA enrichment efficiency (6 ± 6 %) and still much lower than the n-3 PUFA efficiencies
281 obtained with fish oil, *Isochrysis galbana* and DHA gold (respectively 54 ± 5 %, 30 ± 6 %, 45 ±
282 5 %). This means that also the ALA enrichment was rather inefficient in the egg yolk, not only
283 for the n-3 LC-PUFA sources but also for flaxseed. This corresponds with literature, where was
284 observed that the n-3 PUFA were preferentially stored as DHA in the egg yolk (Fredriksson *et*
285 *al*., 2006; Nitsan *et al*., 1999). Only overdosing the amount of flaxseed in the diet of the laying
286 hens leads to a significant higher increase of the ALA and DHA content in the egg (Aymond
287 and Van Elswyk; 1995; Van Elswyk, 1997).

288 **3.4. Zootechnical performance of the laying hens**

289 Globally, no drastic influences of the different n-3 PUFA sources on the zootechnical
290 parameters were observed (**Table 4**). In literature, the influence of feed supplementation on
291 the zootechnical performance parameters are very contradictory for the different sources
292 (Fraeye *et al*., 2012). This can probably be explained by different experimental setups, but, in
293 most cases, also no drastic changes of the zootechnical performance parameters were
294 observed (Fraeye *et al*., 2012).

4. Conclusion

The four different n-3 PUFA sources (flaxseed, *Isochrysis galbana*, fish oil and DHA Gold) supplemented, in such way to reach the same supplemented n-3 PUFA amount, to laying hens led to an increased level of n-3 LC-PUFA in the egg yolk. Mainly DHA enrichment was observed for all the sources. Only for the supplementation with flaxseed, also a significant increase of ALA was observed. However, the obtained level of enrichment was not the same for all n-3 PUFA sources, although the same amount of n-3 PUFA was supplemented. The lowest enrichment (efficiency) (≈ 6%) was observed when flaxseed was supplemented to the diet of the laying hens, this because of the inefficient conversion of ALA to DHA. Drastically higher n-3 LC-PUFA enrichments and enrichment efficiencies were observed with supplementation of n-3 LC-PUFA sources. Fish oil led to the highest efficiency (≈ 55%), followed by DHA Gold (≈ 45%) and *Isochrysis galbana* (≈ 30%). The differences in enrichment efficiency with these sources can be explained by the different bio-accessibility of the n-3 PUFA and the different n-3 PUFA profile of the three sources.

ABBREVIATIONS

n-3 LC-PUFA	Omega-3 long chain polyunsaturated fatty acids
ALA	α-linolenic acid
SDA	Stearidonic acid
EPA	Eicosapentaenoic acid
DPA	Docosapentaenoic acid
DHA	Docosahexaenoic acid

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REFERENCES

Aymond, W.M. & Van Elswyk, M.E. (1995). Yolk thiobarbituric acid reactive substances and n-3 fatty acids in response to whole and ground flaxseed. *Poulty Science, 74*, 1388-1394.

Bean, L. D. & Leeson, S. (2003). Long term effects of feeding flaxseed on performance and egg fatty acid composition of brown and white hens. *Poultry Science*, *82*, 388–394.

Bourre, J. M. (2005). Where to find omega-3 fatty acids and how feeding animals with diet enriched in omega-3 fatty acids to increase nutritional value of derived products for human: what is actually useful. *The Journal of Nutrition, Health and Aging*, *9*, 232–242.

Bovet, P., Faeh, D., Madeleine, G., Viswanathan, B. & Paccaud, F. (2007). Decrease in blood triglycerides associated with the consumption of eggs of hens fed with food supplemented with fish oil. *Nutrition, Metabolism and Cardiovascular Diseases*, *17*, 280–287.

Bruneel, C., Lemahieu, C., Fraeye, I., Ryckebosch, E., Muylaert, K., Buyse, J. & Foubert, I. (2013). Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs. *Journal of Functional Foods*, *32*, 897–904.

Burdge, G. (2004). α-Linolenic acid metabolism in men and women: Nutritional and biological implications. *Current Opinion in Clinical Nutrition and Metabolic Care, 7*, 137-144.

Cachaldora, P., Blas, J. C. D., Álvarez, C. & Méndez, J. (2005). Short communication: Effects of type and level of supplementation with dietary n-3 fatty acids on yolk fat composition and n-3 fatty acid retention in hen eggs. *Spanish Journal of Agricultural Research*, *3*, 209–212.

346 Cachaldora, P., García-Rebollar, P., Alvarez, C., De Blas, J. C. & Méndez, J. (2006). Effect of type
347 and level of fish oil supplementation on yolk fat composition and n-3 fatty acid retention
348 efficiency in laying hens. *British Poultry Science*, *47*, 43–49.

349 Cachaldora, P., García-Rebollar, P., Alvarez, C., Méndez, J. & De Blas, J. C. (2008). Double
350 enrichment of chicken eggs with conjugated linoleic acid and n-3 fatty acids through dietary
351 fat supplementation. *Animal Feed Science and Technology*, *144*, 315–326.

352 Carrillo, S., López, E., Casas, M. M., Avila, E., Castillo, R. M., Carranco, M. E., Calvo, C. & Pérez-
353 Gil, F. (2008). Potential use of seaweeds in the laying hens ration to improve the quality of n-
354 3 fatty acid enriched eggs. *Journal of Applied Phycology*, *20*, 721–728.

355 Caston, L. & Leeson, S. (1990). Research note: Dietary flax and egg composition. *Poultry*
356 *Science, 69*, 1617-1620.

357 Cheng, C. H., Shen, T. F., Chen, W. L. & Ding, S. T. (2004). Effects of dietary algal
358 docosahexaenoic acid oil supplementation on fatty acid deposition and gene expression in
359 laying Leghorn hens. *The Journal of Agricultural Science*, *142*, 683–690.

360 Fraeye, I., Bruneel, C., Lemahieu, C., Buyse, J., Muylaert, K. & Foubert, I. (2012) Dietary
361 enrichment of eggs with omega-3 fatty acids: a review. *Food Research International, 48*, 961-
362 969.

363 Fredriksson, S., Elwinger, K. & Pickova, J. (2006). Fatty acid and carotenoid composition of egg
364 yolk as an effect of microalgae addition to feed formula for laying hens. *Food Chemistry*, *99*,
365 530-537.

366 Gogus, U.& Smith, C. (2010). n-3 omega fatty acids: a review of current knowledge.
367 *International Journal of Food Science and Technology*, *45*, 417–436.

368 Gonzalez-Esquerra, R. & Leeson, S. (2000). Effect of feeding hens regular or deodorized
369 menhaden oil on production parameters, yolk fatty acid profile, and sensory quality of eggs.
370 *Poultry Science*, *79*, 1597–1602.

371 Harris, W. S. (2007). Omega-3 fatty acids and cardiovascular disease: a case for omega-3 index
372 as an new risk factor. *Pharmacological Research : the official journal of the Italian*
373 *Pharmacological Society*, *55*, 217–223.

374 Herber, S.M. & Van Elswyk, M.E. (1996). Dietary marine algae promotes efficient deposition
375 of n-3 fatty acids for the production of enriched shell eggs. *Poultry Science*, *75*, 1501-1507.

376 Herber-McNeill, S. M. & Van Elswyk, M. E. (1998). Dietary marine algae maintains egg
377 consumer acceptability while enhancing yolk color. *Poultry Science, 77*, 493-496.
378 Jeyakumar, H. N. (2012) Health benefits of n-3 polyunsaturated fatty acids: eicosapentaenoic
379 acid and docosahexaenoic acid. In Siriwardhana, N., Kalupahana, N. S. &Moustaid-Moussa
380 (first edition, vol 65), *Advances in Food and Nutrition Research* (pp. 211-222). Elsevier Inc.

381 Jordan, R. G. (2010). Prenatal omega-3 fatty acids: review and recommendations. *Journal of*
382 *Midwifery and Womens' Health*, *55*, 520–528.

383 Komprada, T. (2012). Eicospentaenoic and docosahexaenoic acids as inflammation-
384 modulating and lipid homeostasis influencing nutraceuticals: A review. *Journal of Functional*
385 *Foods*, *4*, 25-38.

386 Kris-Etherton, P. M., Grieger, J. A. & Etherton, T. D. (2009). Dietary reference intakes for EPA
387 and DHA. *Prostaglandins, leukotrienes and Essential fatty acids*, *81*, 99–104.

388 Lawlor, J. B., Gaudette, N., Dickson, T. & House, J. D. (2010). Fatty acid profile and sensory
389 characteristics of table eggs from laying hens diets containing microencapsulated fish oil.
390 *Animal Feed Science and Technology, 156*, 97–103.

391 Lemahieu, C., Bruneel, C., Termote-Verhalle, R., Muylaert, K., Buyse, J. & Foubert, I. (2013a)
392 Impact of feed supplementation with different omega-3 rich microalgae species on
393 enrichment of eggs of laying hens. *Food Chemistry*, 4051–4059.

394 Lemahieu, C., Bruneel, C., Termote-Verhalle, R., Muylaert, K., Buyse, J. & Foubert, I. (2013b).
395 Omega-3 long-chain polyunsaturated fatty acid enriched eggs by microalgal supplementation.
396 *Lipid Technology*, *25*, 204–206.

397 Lemahieu, C., Bruneel, C., Termote-Verhalle, R., Muylaert, K., Buyse, J. & Foubert, I. (2014).
398 Effect of different microalgal n-3 PUFA supplementation doses on yolk color and n-3 LC-PUFA
399 enrichment in the egg. *Algal Research, 6*, 119-123.

400 Meyer, B. J. (2011). Are we consuming enough long chain omega-3 polyunsaturated fatty acids
401 for optimal health? *Prostaglandins, leukotrienes and Essential fatty acids*, *85*, 275–280.

402 Morato, P. N., Rodrigues, J. B., Moura, C. S., e Silva, F. G. D., Esmerino, E. A., Cruz, A. G., Bolin,
403 H.M.A., Amaya-Farfan, J & Lollo, P. C. B. (2015). Omega-3 enriched chocolate milk: A functional
404 drink to improve health during exhaustive exercise. *Journal of Functional Foods, 14*, 676–683.
405

406 Nitsan, Z., Mokady, S. & Sukenik, A. (1999). Enrichment of poultry products with omega3 fatty
407 acids by dietary supplementation with the alga Nannochloropsis and mantur oil. *Journal of*
408 *Agricultural and Food Chemistry*, *47*, 5127-5132.
409 Nuño, K., Villarruel-López, A., Puebla-Pérez, A.M., Romero-Velarde, E., Puebla-Mora, A.G. &
410 Ascencio, F. (2013). Effects of the marine microalgae *Isochrysis galbana* and *Nannochloropsis*
411 *oculata* in diabetic rats. *Journal of Functional Foods*, *5*, 106-115.

412 Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A. & Millington, K. J. (2004) The health benefits of
413 omega-3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition*
414 *and Dietetics : the official journal of the British Dietetic Association*, *17*, 449–459.

415 Ryckebosch, E., Muylaert, K. & Foubert, I. (2012). Optimization of an analytical procedure for
416 extraction of lipids from microalgae. *Journal of the American Oil Chemists'. Society*, *89*, 189–
417 198.

418 Schiedeler, S.E. & Froning, G.W. (1996). The combined influence of dietary flaxseed variety,
419 level, form and storage conditions on egg production and composition among vitamin E-
420 supplemented hens. *Poultry Science*, *75*, 1221-1226.

421 Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *The American*
422 *Journal of Clinical Nutrition*, *70*, 560S–569S.

423 Sioen, I., Pynaert, I., Matthys, C., De Backer, G., Van Camp, J. & De Henauw, S. (2006). Dietary
424 intakes and food sources of fatty acids for Belgian woman, focused on n-6 and n-3
425 polyunsaturated fatty acids. *Lipids*, *41*, 415–422.

426 Sioen, I., De Henauw, S., Van Camp, J., Volatier, J.-L. & Leblanc, J.-C. (2009). Comparison of the
427 nutritional-toxicological conflict related to seafood consumption in different regions
428 worldwide. *Regulatory Toxicolology and Pharmacology: RTP*, *55*, 219–228.

429 Stratulat, I., Britten, M., Salmieri, S., Fustier, P., St-Gelais, D., Champagne, C. P., & Lacroix, M.
430 (2015). Enrichment of cheese with vitamin D3 and vegetable omega-3. *Journal of Functional*
431 *Foods*, *13*, 300–307.

432 Trautwein, E. A. (2001). n-3 fatty acids: physiological and technical aspects for their use in
433 food. *Sciences-New York*, *103*, 45–55.

434 Van Elswyk, M.E., Dawson, P.L. & Sams, A.R. (1995). Dietary menhaden oil influences sensory
435 characteristics and headspace volatiles of shell eggs. *Journal of Food Science, 60,* 85-89.

436 Van Elswyk, M. E. (1997). Comparison of n-3 fatty acid sources in laying hen rations for
437 improvement of whole egg nutritional quality: a review. *The British Journal of Nutrition*, *78*,
438 S61-69.

439 Wootton, E. C., Zubkov, M. V., Jones, D. H., Jones, R. H., Martel, C. M., Thornton, C. A. &
440 Roberts, E. C. (2007). Biochemical prey recognition by planktonic protozoa. *Environmental*
441 *Microbiology*, *9*, 216–222.

442 Yashodhara, B. M., Umakanth, S., Pappachan, J. M., Bhat, S. K., Kamath, R. & Choo, B. H.
443 (2009). Omega-3 fatty acids: a comprehensive review of their role in health and disease.
444 *Postgraduate Medical Journal*, *85*, 84–90.

445 Zhu, C. J. & Lee, Y. K. (1997). Determination of biomass dry weight of marine microalgae.
446 *Journal of. Applied. Phycology*, *9*, 189–194.

447

448 **TABLES**

449 **Table 1: n-3 PUFA content (% of the biomass; mean ± SD; n=3) of the four n-3 PUFA sources:**

450 **extruded flaxseed, *Isochrysis galbana*, fish oil and DHA Gold.**

	Flaxseed	*Isochrysis*	Fish oil	DHA Gold
ALA	21.4 ± 0.7	1.36 ± 0.03	1.105 ± 0.003	0.019 ± 0.0002
SDA	-	2.67 ± 0.07	1.35 ± 0.03	0.093 ± 0.009
EPA	-	0.082 ± 0.005	7.06 ± 0.15	0.36 ± 0.04
DPA	-	0.022 ± 0.002	1.34 ± 0.05	0.121 ± 0.010
DHA	-	1.78 ± 0.04	6.76 ± 0.16	26.4 ± 0.3

451

Table 2: Level of the different n-3 PUFA (ALA, EPA, DPA and DHA, in mg/egg, mean ± SD, n=8) in the egg at the end of the supplementation period obtained by feeding with extruded flaxseed, *Isochrysis galbana*, fish oil and DHA Gold.

	ALA	EPA	DPA	DHA
Control	9.9 ± 1.0[a]	-	3.0 ± 0.5[a]	21 ± 3[a]
Flaxseed	19.2 ± 2.5[c]	0.05 ± 0.15[a]	5.5 ± 3.0[b]	33 ± 4[b]
Isochrysis	12.6 ± 1.2[b]	0.94 ± 0.15[c]	5.9 ± 1.0[b]	67 ± 3[c]
Fish oil	11.6 ± 1.2[b]	3.5 ± 0.3[d]	10.0 ± 1.1[c]	92 ± 3[d]
DHA Gold	12.6 ± 0.9[b]	0.6 ± 0.3[b]	3.2 ± 0.6[a]	90 ± 5[d]

Within each n-3 PUFA, results with the same letter for the different sources are not significantly different ($p < 0.05$).

Table 3: Incorporation efficiency of the n-3 LC-PUFA in the egg yolk for the supplementation of the four different n-3 LC-PUFA sources (in %, mean ± SD, n=8): Flaxseed, *Isochrysis galbana*, fish oil and DHA Gold. The incorporation efficiency is calculated by taking the ratio of the enrichment of n-3 LC-PUFA in the egg (in mg mean ± SD, n=8) to the actual n-3 PUFA intake (in g; mean ± SD, n=8), multiplied with 100.

	Actual n-3 PUFA intake (mg)	Enrichment of n-3 LC-PUFA (mg)	Enrichment efficiency (%)
Flaxseed	140 ± 2	8 ± 8[a]	6 ± 6[a]
Isochrysis	143.0 ± 0.2	43.4 ± 0.2[b]	30 ± 4[b]
Fish oil	138 ± 2	76 ± 5[d]	55 ± 4[d]
DHA Gold	141.1 ± 1.2	64 ± 6[c]	45 ± 5[c]

Results with the same letter in the same column are not significantly different ($p < 0.05$)

Table 4: Zootechnical performance parameters and egg quality parameters for the four n-3 PUFA sources during the supplementation period: feed intake (in g, mean ± SD; n = 8), egg production rate (in %), egg weight (in g, mean ± SD, n=8) and yolk weight (in g, mean ± SD; n = 8).

n-3 PUFA source	Feed intake (g)	Egg production rate (%)	Egg weight (g)	Yolk weight (g)
Control	118.3 ± 0.6^{bc}	98.0	59.8 ± 1.2^{ab}	14.5 ± 1.3^{a}
Flaxseed	116.5 ± 2.0^{a}	97.6	62.1 ± 1.3^{c}	15.1 ± 0.5^{a}
Isochrysis	119.2 ± 0.2^{c}	97.6	58.9 ± 1.2^{a}	14.4 ± 1.3^{a}
Fish oil	118.0 ± 0.6^{ab}	99.2	58.7 ± 1.0^{a}	14.8 ± 1.0^{a}
DHA Gold	117.6 ± 1.0^{ab}	98.2	60.7 ± 0.9^{bc}	15.5 ± 1.2^{a}

Results with the same letter in the same column are not significant different ($p < 0.05$)