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Moulay Sahaka, Sawsan Amara, Jutarat Wattanakul, Mohamed Gedi, Noelia Aldai, et al.. The digestion of galactolipids and its ubiquitous function in Nature for the uptake of the essential  $\alpha$ -linolenic acid. *Food and Function*, 2020, 11 (8), pp.6710-6744. 10.1039/d0fo01040e . hal-02903683

**HAL Id: hal-02903683**

**<https://hal.science/hal-02903683v1>**

Submitted on 13 Nov 2020

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**The digestion of galactolipids and its ubiquitous function in Nature  
for the uptake of the essential  $\alpha$ -linolenic acid**

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## **Abstract**

Galactolipids, mainly monogalactosyl diglycerides and digalactosyl diglycerides are the main lipids found in the membranes of plants, algae and photosynthetic microorganisms like microalgae and cyanobacteria. As such, they are the main lipids present at the surface of the earth. They may represent up to 80 % of the fatty acid stocks, including a large proportion of polyunsaturated fatty acids mainly  $\alpha$ -linolenic acid (ALA). Nevertheless, the interest in these lipids for nutrition and other applications remains overlooked, probably because they are dispersed in the biomass and are not as easy to extract as vegetable oils from oleaginous fruit and oil seeds. Another reason is that galactolipids only represent a small fraction of the acylglycerolipids present in modern human diet. In herbivores such as horses, fish and folivorous insects, galactolipids may however represent the main source of dietary fatty acids due to their dietary habits and digestion physiology. The development of galactolipase assays has led to the identification and characterization of the enzymes involved in the digestion of galactolipids in the gastrointestinal tract, as well as by microorganisms. Pancreatic lipase-related protein 2 (PLRP2) has been identified as an important factor of galactolipid digestion in humans, together with pancreatic carboxyl ester hydrolase (CEH). The levels of PLRP2 are particularly high in monogastric herbivores thus highlighting the peculiar role of PLRP2 in the digestion of plant lipids. Similarly, pancreatic lipase homologs are found to be expressed in the midgut of folivorous insects, in which a high galactolipase activity can be measured. In fish, however, CEH is the main galactolipase involved. This review discusses the origins and fatty acid composition of galactolipids and the physiological contribution of galactolipid digestion in various species. This overlooked aspect of lipid digestion ensures not only the intake of ALA from its main natural source, but also the main lipid source of energy for growth of some herbivorous species.

**Keywords:** Galactolipase; DGDG; Lipids; MGDG; PUFA

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## **1. The origin of galactolipids and their distribution in plants and other photosynthetic organisms**

Monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), sulfoquinovosyldiacylglycerols (SQDG) (Figure 1), as well as phosphatidylglycerol (PG) are the main lipids found in the chloroplast membranes and galactolipids account for as much as 70-80% of the total lipids in plant cells <sup>1</sup>. The chloroplast is a specific organelle of photosynthetic cells (Figure 2), that originates from the primary endosymbiosis of a cyanobacterium by a eukaryotic host more than 1 billion years ago. This event subsequently gave rise to green algae and plants (containing “chloroplasts”) and red algae (containing “rhodoplasts”) and several other photosynthetic eukaryotic lineages, such as diatoms, resulting from secondary and tertiary endosymbioses of free-living photosynthetic eukaryotes by eukaryotic hosts <sup>2, 3</sup>. The inner envelope of the chloroplast and the thylakoid membranes, where photosynthesis occurs, have a similar lipid composition in MGDG, DGDG, SQDG and PG, while the outer envelope also contains phosphatidylcholine (PC), which is typical of eukaryotic cells (Figure 3). Plants like spinach contain a portion of galactolipids (59.5%) much larger than that of phospholipids (22.4%) among their polar lipids <sup>4</sup>. In the alga *Chlorella*, galactolipids are four times more abundant than phospholipids <sup>5</sup>. Galactolipids are also much more abundant in the plant kingdom than triacylglycerols (TAG), the main lipid molecules found in the vegetable oils produced from oil seeds (soybean, rapeseed, sunflower, maize, and peanut) and oleaginous fruit (palm, olive). Based on their high proportion in chloroplastic membranes (Figure 3) and the abundance of plants and algae on Earth, it was estimated that galactolipids represent the most abundant acyl lipid class in Nature <sup>6</sup>, and therefore, the main reservoir of fatty acids (FA; 80% vs. 20% in phospholipids and vegetable

oils)<sup>5, 7</sup>. It has been estimated that the surface of membranes made of galactolipids in each square meter (m<sup>2</sup>) of leaves is equal to 3 soccer stadiums or 2.5 hectares<sup>3</sup>. This is made possible by the dense packing of galactolipid bilayers in thylakoid membranes, which results from galactosyl headgroup interactions between bilayers. The hydroxyl groups of galactose form multiple small electric dipoles, that induce a higher repulsion of water molecules, as compared to what occurs in between zwitterionic phospholipid membranes<sup>8, 9</sup>.

Most of the membrane lipids in the chloroplast are assembled in the envelope membranes (Figure 3)<sup>10</sup>. The building blocks for chloroplast glycerolipids are phosphatidic acid (PA) and diacylglycerol (DAG). PA is produced both in chloroplasts and in the endoplasmic reticulum (ER), depending on the plant species. The DAG backbone for galactolipid synthesis can be derived from two different pathways, the prokaryotic pathway localized in the inner membrane of chloroplast and the eukaryotic pathway localized in the ER<sup>11</sup>. These pathways can be distinguished by the FA specificity of the sn-2 lysophosphatidic acid-acyltransferases (LPAAT) involved. The chloroplast-localized enzyme has a strong preference for C16 fatty acids, whereas the ER-localized enzyme has a high specificity for C18 FAs. Thus, a C16 FA at the sn-2 position is a signature for the chloroplastic origin of a DAG backbone. In some higher plants, like *Arabidopsis thaliana* and spinach (*Spinacia oleracea*), chloroplast galactolipids contain a high proportion of 16:3 n-3 at the sn-2 position, which indicates a plastidial pathway for synthesis of these galactolipids. These plants are thus referred to as 16:3 plants, whereas other plants not using the plastidial pathway for plastid galactolipid synthesis are referred to as 18:3 plants<sup>10</sup>. The diacyl precursors derived from ER-localized lipid biosynthesis are transported to the chloroplast envelope by a mechanism that is still unclear<sup>12, 13</sup>. PC, PC-derived DAG, PA, and lyso-phosphatidylcholine (LPC) have all been implicated in ER-to-chloroplast lipid transfer. However, a recent fluxomic study in *Arabidopsis thaliana* mutants devoid of the major chloroplastic lysophospholipid

acyltransferases LPCAT1 and LPCAT2 has narrowed down the ER-to-chloroplast lipid trafficking candidates <sup>14</sup>. LPC was predominantly involved in the incorporation into PC of nascent fatty acids exported from the chloroplast to ER (PC acyl editing), while eukaryotic galactolipid synthesis was not impaired by LPCAT mutations and used a PC pool distinct from that of PC acyl editing.

MGDG synthases catalyze the first step of galactolipid synthesis by transferring one galactose from uridine diphosphate (UDP)-galactose (UDP-Gal) onto DAG (Figures 3 and 4) <sup>15</sup>. MGD1, a type A MGDG synthase localized in the inner envelope of the chloroplast, is highly expressed in green tissues and upregulated by light, whereas MGD2 and MGD3 are type B MGDG synthases, localized to the outer envelope. Expression of MGD2 and MGD3 is observed only in specific organs and they are involved in galactolipid synthesis for extraplastidial membranes and nongreen tissues <sup>16-18</sup>. The upregulation by light of MGDG synthesis by MGD1 has been illustrated by the higher MGDG contents in photoautotrophic (light; CO<sub>2</sub> as the sole source of carbon) versus heterotrophic plant cell cultures (no light; sucrose as the sole source of carbon) <sup>19</sup>.

The second step of galactolipid synthesis is catalyzed by DGDG synthases DGD1 and DGD2, which transfer a galactose from UDP-Gal onto MGDG (Figures 3 and 4) <sup>20</sup>. These enzymes form  $\alpha$ -linkages of the terminal galactose leading to the unique head group structure of DGDG (Figure 1). DGD1, localized in the outer envelope, is responsible for the synthesis of the bulk of DGDG found in chloroplasts. DGD2, which is poorly expressed in leaves, is involved in galactolipid synthesis for extraplastidial membranes and nongreen tissues <sup>21 22</sup>.

The sulfoquinovose polar head of SQDG (Figure 1) is formed from UDP-glucose by condensation with a molecule of sulfite (SO<sub>3</sub><sup>2-</sup>) by SQD1, an UDP-sulfoquinovose synthase <sup>23</sup>. Transfer of sulfoquinovose to DAG is then achieved by an UDP-sulfoquinovose:DAG sulfoquinovosyltransferase (SQD2) forming SQDG (Figure 3) <sup>24</sup>.

The MGDG and DGDG contents of some vegetables, legumes and fruit are given in Table 1.

Apart from leaves, galactolipids are also found in non-photosynthetic parts of plants, like in seeds. In wheat flour, the total content of galactolipids range from 100 to 200 mg per 100 g flour and DGDG and MGDG represent around 77% of these galactolipids<sup>25</sup>. On the contrary to what is found in leaves, DGDG exceeds MGDG in seeds and represents around 60% of total galactolipids (Table 2). It is worth noting the presence of mono- and di-galactosylmonoacylglycerols in flour (Table 2) which indicates that a galactolipase activity is expressed in mature or maturing wheat endosperm<sup>26</sup>. Spring wheat flours are about 17% richer in total lipids, especially due a greater concentration of non-polar lipids in the endosperm of these wheats<sup>25</sup>. Galactolipids are mainly found in the membrane of amyloplasts, and thus the number and size of starch granules can influence their amounts<sup>27</sup>. In oat (*Avena sativa* L.), seed kernels contain much higher polar lipid concentrations than other plant tissues. While total lipid represent  $\approx 8.3\%$  on a dry weigh (DW) basis, 10% are phospholipid and 11% are galactolipids with up to 400 mg DGDG per 100 g of oat flour<sup>28</sup>. Oat galactolipids are characterized by a high molecular diversity, with di-, tri- and tetra-galactosyldiacylglycerols, along with their mono-, di-, and tri-acyl estolides<sup>28,29</sup>.

Galactolipids are also found in the fresh latex from *Hevea brasiliensis*, in which they represent as much as 30% w/w of total lipids. DGDG (84-86%) and MGDG (14-16%) are the main molecular species while no SQDG is found in latex<sup>30</sup>. These galactolipids are characterized by a high content in furan FAs (see section 2.).

## **2. The fatty acid composition of galactolipids**

A unique feature of chloroplast galactolipids is their natural enrichment in long chain trienoic polyunsaturated fatty acids (PUFA)<sup>12, 13</sup> such as  $\alpha$ -linolenic acid (ALA; 18:3 n-3)



which can account for up to 95% of total FAs in some higher plant galactolipids, and hexadecatrienoic acid (16:3 n-3) which can be found in high amounts in MGDG. This peculiar FA composition is tightly related to photosynthesis<sup>5</sup> and the fact that a major part of FA biosynthesis and desaturation occurs in the chloroplast (Figures 3 and 4). Although, chloroplasts are characteristic of plant and green algal eukaryotic cells, they still exhibit many prokaryotic features, such as the synthesis of FAs that occurs in the stroma of the plastid through the action of the Fatty Acid Synthase (FAS) complex. FAS requires a pool of acetyl-CoA as the starting unit<sup>31</sup>, further converted in acyl chains by sequential condensation of two-carbon units provided by malonyl- acyl carrier protein (ACP) at each step of elongation by 3-ketoacyl-ACP synthase I (KASI). KASI allows the synthesis of saturated acyl chains with length up to 16 carbon atoms. 16:0 bound to ACP can be further elongated and converted in 18:0-ACP by an additional cycle of two-carbon condensation catalyzed by a different enzyme (KASII). 18:0-ACP is then converted in 18:1-ACP by stearoyl-ACP  $\Delta$ 9-desaturase (SAD), a soluble enzyme found in the plastid stroma<sup>32, 33</sup>. Then, acyl chains bound to acyl carrier protein (ACP) can either follow i) an incorporation into acyl-glycero-lipids inside the chloroplast via the so-called "prokaryotic" pathway<sup>34</sup> or ii) an export outside the chloroplast to reach the "Eukaryotic" pathway in the ER (Figure 3). The two pathway hypothesis was enunciated by Roughan and Slack<sup>35</sup> after a decade of studies of FA biosynthesis in plants. The export outside the chloroplast requires that 16:0 and 18:1 FAs are released from acyl-ACP by acyl-ACP thioesterases and converted in acyl-CoA in the ER prior to their incorporation in acyl lipids by various acyl transferases. Approximately 40% of FAs synthesized in chloroplasts enter the prokaryotic pathway, whereas 60% are exported to the eukaryotic pathway. About half of these exported FAs return to the plastid after their desaturation in the ER (Figure 4) and are then used for the synthesis of the thylakoid membrane galactolipids<sup>36</sup>.

Desaturation of FAs is tightly linked with the location of the diacyl-glycero-lipids in which they are incorporated and their transfer between the chloroplast envelope and the ER. Extrplastidic FA desaturation mainly occurs on acyl chains esterified to PC (Figure 4)<sup>37</sup> and involves the FA desaturases FAD2<sup>38</sup> and FAD3<sup>39, 40</sup> that convert PC-bound oleate to linoleate ( $\omega$ -6 desaturase) and then to linolenate ( $\omega$ -3 desaturase), respectively. Acyl lipids synthesized in the chloroplast envelope are subject to further desaturation by envelope or thylakoid-bound desaturases like FAD 6 and FAD 8<sup>41</sup>. These desaturases are responsible for the typical FA desaturation signature of plastid lipid, including hexadecatrienoic acid (16:3 n-3) which is generally considered as exclusively plastidial. Plastidic FA desaturation also occurs on acyl chains esterified to MGDG. A unique feature of the biosynthesis of PUFAs in plants is that it increases with the photosynthetic activity<sup>42</sup>. Indeed, a light-dependent regulation of  $\omega$ -3 FAD gene expression was shown in soybean cell cultures, both for FAD acting in the ER like FAD3 and the chloroplast like FAD8<sup>43</sup>. In plant cell cultures grown under various conditions of light, i.e. heterotrophy (no light; sucrose as the sole source of carbon), and photoautotrophy (light; CO<sub>2</sub> as the sole source of carbon), the ALA content in polar lipids are increasing with light exposure and the FA composition of photoautotrophic cells is similar to that of leaves. Conversely, the FA composition of heterotrophic cells (no light) is similar to that of seeds with low levels of PUFA and increased levels of saturated FAs (SFA; 16:0 and 18:0)<sup>19, 44</sup>. These light-associated features of FA composition in polar lipids are also found in neutral lipids from plant cell cultures, leaves and seeds<sup>45</sup>, supporting the role of TAGs in the temporary storage of FAs for membrane lipid synthesis<sup>46</sup>.

We have seen that the prokaryotic and eukaryotic pathways form diacyl precursors of galactolipids with only C16 and C18 FAs at the sn-2 position of the DAG backbone respectively. Typically, in leaves, 18:3 n-3/16:3 n-3 MGDG and 18:3 n-3/ 16:0 DGDG are the predominant molecular species of prokaryotic galactolipids while 18:3 n-3/18:3 n-3 MGDG

and 18:3 n-3/18:3 n-3 DGDG are the most represented eukaryotic galactolipids (Figure 1) <sup>47</sup>. As an example, the FA composition and region-distribution in MGDG and DGDG from spinach leaves is given in Table 3.

In contrast to what is found in galactolipids from leaves, the FA composition of galactolipids in seeds is characterized by high levels of linoleic acid (LA) and low levels of ALA such as in wheat (Table 2). The main molecular species of galactolipids in wheat flour are 18:2 n-6/18:2 n-6 DGDG, 16:0/18:2 n-6 DGDG, 18:2 n-6/18:2 n-6 MGDG, and 16:0/18:2 n-6 MGDG <sup>48</sup>. In oat seeds, DGDG contain significant amounts of an unusual hydroxy acid, (15 R)-hydroxy-(9Z),(12Z)-octadecadienoic acid or avenoleic acid <sup>49</sup>.

In latex from *Hevea brasiliensis*, MGDG and DGDG do not contain high amounts of ALA as found in leaves of Euphorbiaceae but they are characterized by high amounts of an oxidized C18 FA with a methylated furan ring. This furan FA (FuFA; 10,13-epoxy-11-methyl octadeca-10,12-dienoic acid) is the product of LA oxidation by a multi-step process involving lipoxigenase. It represents 42% of total FAs in latex glycolipids, with FuFA/FuFA MGDG and FuFA/FuFA DGDG being the most represented (around 40%) molecular species of galactolipids <sup>30</sup>. FuFA is characterized by a strong antioxidant activity <sup>50,51</sup>.

Since algae and microalgae may represent novel sources of galactolipids and PUFA of nutritional interest <sup>52</sup>, it is worth mentioning here that galactolipids from algae differ from those of plants by their enrichment in very long chain PUFAs (VLC-PUFA) such as arachidonic acid (ARA; 20:4 n-6), eicosapentaenoic acid (EPA; 20:5 n-3) or docosahexaenoic acid (DHA; 22:6 n-3). These FAs with chain lengths of 20 carbons and over result from a combination of elongation and desaturation reactions taking place in the ER <sup>53</sup>. The so-called  $\Delta$ 6-pathway is the most common for producing VLC-PUFA from LA or ALA in algae <sup>54</sup>. It involves  $\Delta$ 6-desaturase,  $\Delta$ 6-elongase and  $\Delta$ 5-desaturase activities to produce ARA or EPA,

respectively<sup>55, 56</sup>. Further elongation to 22-carbon chain length products, docosapentaenoic acid (DPA 22:5 n-6) or DHA, uses a  $\Delta$ 5-elongase and a  $\Delta$ 4-desaturase.

In freshwater algae of the Glaucocystophyte genera, it was found that the major forms of MGDG and DGDG (with sn-1/sn-2 regiochemistry) are enriched in EPA and are 20:5 n-3/16:0 MGDG, 20:5 n-3/20:5 n-3 MGDG, 20:5 n-3/16:0 DGDG, and 20:5 n-3/20:5 n-3 DGDG<sup>57</sup>. In the marine diatom *Phaeodactylum tricornutum*, the major forms of galactolipids are 16:1/16:0 SQDG, 20:5 n-3/16:3 n-3 MGDG and 20:5 n-3/16:2 DGDG<sup>58</sup>. The Haptophyta/Prymnesiophyta *Isochrysis galbana* microalga is distinguished from most of the other microalgae by high levels of DHA in galactolipids<sup>59</sup>. In the red microalgae, *Porphyridium aeruginum*, galactolipids are enriched in molecular species with ARA and EPA (20:4 n-6/16:0 SQDG, 20:5 n-3/16:0 SQDG, 20:4 n-6/18:3 n-3 MGDG, 20:4 n-6/16:0 MGDG)<sup>60, 61</sup>. Metabolic engineering of microalgae like *Phaeodactylum tricornutum* now allows to increase the production of DHA through the heterologous expression of  $\Delta$ -5elongase and  $\Delta$ 6-desaturase<sup>62</sup>.

Several studies have been dedicated to the positional distribution of FAs in galactolipids, using either microbial lipases<sup>63</sup>, pancreatic extracts<sup>64-66</sup> or purified pancreatic lipase-related protein 2<sup>67</sup> (see Table 3 for distribution in galactolipids from spinach leaves). In all cases, these analyses were based on the use of galactolipases with a regioselectivity for the sn-1 position of the glycerol backbone in galactolipids (Figure 5).

For additional and more detailed informations on FA and galactolipid biosynthesis in plants, see the excellent review by Hölzl and Dormann<sup>68</sup>, the Arabidopsis Book<sup>10</sup> and the associated website ARALIP (<http://aralip.plantbiology.msu.edu/>), as well as the AOCS Lipid Library (<https://lipidlibrary.aocs.org/chemistry/physics/plant-lipid/50-years-of-galactolipid-research-the-beginnings>).

### 3. Galactolipases or galactoglycerolipid acyl hydrolases

In March 2020, a survey of the literature on lipolytic enzymes using PubMed gave 50 articles accessible with the “galactolipase” keyword whereas 43826 and 61079 articles included “lipase” and “phospholipase” keywords, respectively. A more focussed search for galactoglycerolipid acyl hydrolase (EC 3.1.1.26; Figure 5) revealed several additional papers dealing with the degradation of plant lipids and partial characterization of plant galactolipases, but overall, this category of enzymes, their physiological roles and potential applications remain poorly investigated. Although the existence of galactoglycerolipid acyl hydrolases in various extracts of plant leaves (runner bean, kidney bean, spinach) has been known for a long time <sup>69</sup>, only a few of these enzymes have been isolated and biochemically characterized, mainly because their specific activities on exogeneous substrates are usually low in natural sources and purification requires a drastic enrichment. Galactolipases were however purified from potatoes <sup>70</sup> and kidney beans <sup>71</sup>. These enzymes have a broad substrate specificity towards glycolipids, phospholipids, and monoacylglycerols, but display no or weak activity towards TAGs. The use of molecular biology tools has allowed a better characterization of plant galactolipases. For instance, a cDNA (Vupat1) encoding a predicted 43 kDa protein and showing homology with patatin, a potato tuber storage protein with lipolytic acyl hydrolase activity, was isolated from drought-stressed cowpea (*Vigna unguiculata*) leaves. The recombinant protein VUPAT1 expressed in the baculovirus system displayed preferentially galactolipid acyl hydrolase activity, while phospholipids were very slowly hydrolyzed and apparently TAGs were not deacylated <sup>72</sup>. These properties were therefore similar to those reported for native galactolipases. Highly thermostable galactolipase activities were also identified in spinach and carrot, and further used as indicators for the determination of lipid degradation during the storage of vegetables <sup>4</sup>. Upon wounding of plant tissues, short-chain aldehydes such as (3Z)-hexenal and n-hexanal are known to be formed

from galactolipid PUFA through the sequential actions of galactolipase, lipoxygenase and fatty acid hydroperoxide lyase activities <sup>73</sup>. Similar defence processes were observed in diatoms (*Thalassiosira rotula*) with the production of antiproliferative aldehydes (2,4-decadienal and 2,4,7-decatrienal) after cell damage or upon wounding <sup>74, 75</sup>. Patatin-like lipolytic acyl hydrolases involved in galactolipid metabolism in the marine diatom *Pseudo-nitzschia* have been identified <sup>76</sup>.

In the microalgae *Chlamydomonas reinhardtii*, it has been shown that a galactolipase designated as plastid galactoglycerolipid degradation 1 (PGD1) is involved in lipid remodelling and allows the use of FAs initially incorporated in chloroplast membrane lipids for the synthesis of TAGs <sup>77</sup>. An enzyme (ckGL) with galactolipase activity on DGDG>MGDG>SQDG and phospholipase A1 activity on PG was also identified and cloned in the microalga *Chlorella kessleri* <sup>78</sup>. Both microalgal galactolipases show sequence homology with fungal lipases of the lipase 3 family (see below).

The galactolipase activity of some microbial lipases has also been known for a long time. For instance, the lipase from the fungus *Rhizopus arrhizus* (or *Rhizopus Orizae*) has been commonly used since 1973 to study the FA composition and distribution in galactolipids because it has specificity for the hydrolysis of ester bond at the sn-1 position of galactolipids <sup>63</sup>. Several microbial lipases with galactolipase activity have been identified <sup>79-81</sup> but little is known about their physiological function in microorganisms. In phytopathogens like the fungus *Fusarium solani*, several enzymes with galactolipase activity were found <sup>80</sup>, including the well characterized cutinase <sup>79</sup>, and one can assume that these enzymes are involved in the degradation of the lipid barrier and membranes of plant tissues. It was also shown that galactolipase activity from the gut microbiota is involved in the ruminal hydrolysis of grass lipids. Using autoclaved grass to inactivate plant galactolipases, it was observed that <sup>14</sup>C-labelled grass galactolipids are rapidly metabolized with the release of <sup>14</sup>C-labelled FAs when

grass is homogenized with sheep rumen contents but are not metabolized when the rumen content has been boiled to destroy microbial enzymes. It was concluded that in the sheep the lipases of rumen micro-organisms play a major role in the ruminal digestion of the complex lipids mixture present in grass and that plant galactolipids could be fully digested by a process involving both microbial enzymes and endogenous galactolipases naturally present in plant leaves<sup>82</sup>. Since then, little attention has been paid to galactolipid digestion in non-ruminant mammals, probably because galactolipids were not considered as important lipids in the diet of these animals nor humans.

Studies on the structure-function relationships of galactolipases have however emerged from the characterization of mammalian galactolipases, especially pancreatic lipase-related protein 2 (PLRP2), with the obtention of several enzyme 3D structures<sup>83-86</sup> and the development of new galactolipase assays using synthetic medium chain galactolipids either in the form of monomolecular films<sup>87</sup> or in the form of mixed micelles with bile salts<sup>88</sup>. Most galactolipases (EC 3.1.1.26) characterized so far, like PLRP2, have broad substrate specificity, the opposite to the so-called “true lipases” that display high activity and selectivity towards TAG and DAG (for instance gastric and classical pancreatic lipases). They often display phospholipase A1 activity (EC 3.1.1.32)<sup>79-81, 89</sup> on various phospholipids<sup>90</sup>, and can usually hydrolyze most acyl glycerols *in vitro*, including TAG which also ranks them as triacylglycerol hydrolases (EC3.1.1.3) or lipases. Their ability to accommodate substrates with various chemical groups at the *sn*-3 position of the glycerol backbone can be partly explained by a larger, more accessible and more hydrophilic catalytic crevice compared to true lipases (Figure 7). This is sometimes due to the absence of the lid controlling the access to the active site of lipase such as in guinea pig PLRP2 (GPLRP2; Figures 6 and 7B) and *Fusarium solani* cutinase<sup>91</sup> (Figures 7C). Alternatively, the lid can be present but found in a conformation that allows a full access to the active site such as in Human PLRP2<sup>84</sup>

(HPLRP2; Figures 6 and 7A). This “open” conformation generates a large cavity capable of accommodating the digalactose polar head of DGDG, similar to that observed in the active site of GPLRP2 (Figure 7B), as well as of *Fusarium solani* cutinase (Figure 7C), but absent from the classical human pancreatic lipase (HPL) <sup>85</sup>. Molecular docking of a 18:3 n-3/18:3 n-3 DGDG molecule in the active site of HPLRP2, GPLRP2 and cutinase reveals a similar topology for the binding sites of the digalactosyl polar head (*sn*-3 position) and the acyl chain found at the *sn*-1 position, that closely interact with apolar residues of the  $\beta$ 9-loop in PLRPs. The  $\beta$ 9-loop has an essential contribution to the stabilization of the acyl enzyme intermediate formed during the lipolysis reaction <sup>92</sup>.

Almost all mammalian and fungal galactolipases characterized so far, for which the 3D structure is known, belong to the structural family of  $\alpha/\beta$  hydrolases, like lipases. They are serine enzymes and their activities are based on a serine-histidine-aspartic/glutamic acid catalytic triad. Figure 8A shows the structural superimposition of HPLRP2 and human pancreatic carboxyl ester hydrolase/bile-salt stimulated lipase (HCEH/BSSL), another digestive lipase with galactolipase activity. Although these lipases belong to distinct gene families and have no sequence homology, they share the  $\alpha/\beta$  hydrolase fold with the active site serine located in the so-called “nucleophilic elbow” <sup>93</sup>. Molecular docking of a 18:3 n-3/18:3 n-3 DGDG molecule in the active site of HCEH 3D structure also reveals the presence of a large cavity capable of accommodating the digalactose polar head of DGDG (Figure 9A), similar to that observed in PLRPs and cutinase (Figure 7). The docking of DGDG in the active site of bovine CEH (BCEH) 3D structure is not impaired by the presence of bile salt molecules co-crystallized with BCEH and located near the digalactosyl binding site (Figure 9B). The resulting 3D model suggests that both the substrate and bile salt monomers can bind to the enzyme upon hydrolysis of mixed galactolipid-bile salt micelles (see below).



The microalgal galactolipase PGD1 identified in *Chlamydomonas reinhardtii* (NCBI Sequence XP\_001693105.1) is homologous to *Thermomyces lanuginosus* lipase and similar fungal lipases from the lipase 3 family, and therefore also predicted to be an  $\alpha/\beta$  hydrolase. The plant galactolipase VUPAT1 from *Vigna unguiculata* shows however homology with patatin, a potato tuber storage protein with non-specific lipid acyl hydrolase activity and a serine-aspartic catalytic dyad<sup>94</sup>. This type of galactolipase is therefore more closely related to cytosolic phospholipase A2<sup>95</sup> than to lipases.

Apart from these structural features, what mainly differentiates enzymes with galactolipase activity from true lipases is their preference for substrates forming small aggregates dispersed in solution like monoglycerides, phospholipids and galactolipids that form mixed micelles in the presence of bile salts, whereas true lipases show a higher activity on insoluble substrates forming oil-in-water emulsions like TAG and DAG. The recognition of the lipid-water interface therefore plays an essential role in the substrate specificity of galactolipases. Using monomolecular films of MGDG and DGDG, enzymes having an amphiphilic lid controlling the access to the active site like the lipase from *Thermomyces lanuginosus* (TLL) and HPLRP2 were found to act at higher surface pressures than those with no lid like cutinase and GPLRP2<sup>79</sup>. The presence of a lid allows a faster activity on monomolecular films of MGDG, which suggest a faster adsorption at the lipid-water interface. This finding suggests that galactolipases with a lid might have a more efficient binding to membranes such as the plant thylakoid membranes.

The highest galactolipase activities have however been measured when galactolipids are mixed with bile salts like sodium taurodeoxycholate (NaTDC) or sodium taurocholate (NaTC), which suggests that an efficient galactolipid digestion in the gastrointestinal tract, requires the solubilization of plant membranes by bile salts and the dispersion of galactolipids in the aqueous phase<sup>88</sup>. The hydrolysis of galactolipid mixed micelles depends on the bile salt

to galactolipid molar ratio and shows an optimum that depends on the substrate (MGDG or DGDG) and the acyl chain length of the substrate<sup>67, 88</sup>. For instance, GPLRP2 and HPLRP2 were found to display optimum activities at NaTDC to galactolipid molar ratios of 1.3, 0.5 and 0.25 on mixed micelles of NaTDC/medium chain-MGDG, NaTDC/long chain-DGDG and NaTDC/medium chain-DGDG, respectively<sup>67</sup>. It is worth mentioning that these conditions were also found to be optimum for other enzymes like human and bovine pancreatic CEH<sup>88</sup>, *Thermomyces lanuginosus* lipase<sup>79</sup>, *Fusarium solani* cutinase<sup>79</sup>, *Fusarium solani* (phospho)lipase<sup>80</sup> and *Talaromyces thermophilus* lipase<sup>81</sup> (Table 4) which suggests that all these enzymes preferentially recognize a specific organization and presentation of galactolipids in mixed micelles. Mixed micelles made of NaTDC and medium chain galactolipids were characterized by dynamic light scattering at 37 °C and pH 8, and their hydrodynamic diameters (z-average) were found to be  $9.4 \pm 0.3$  nm for NaTDC/di-octanoyl-MGDG mixed micelles at a molar ratio of 1.33 and  $24.5 \pm 0.2$  nm for NaTDC /di-octanoyl-DGDG mixed micelles at a molar ratio of 0.25<sup>81</sup>.

Nevertheless, when various substrates under optimum micellar conditions were tested with human and guinea pig PLRP2s, the length of acyl chains and the size of the galactosyl polar head (mono- or di-galactosyl) of the galactolipid were not found to have major effects on the specific activities of PLRP2s, which were found to be very high on all substrates (from  $1756 \pm 208$  to  $5420 \pm 85$  U/mg, with 1 U = 1  $\mu$ mole of fatty acid released per min) (Table 4)<sup>67</sup>.

#### **4. Hydrolysis of galactolipids by pancreatic lipolytic enzymes**

It had been shown in the 1970s, that MGDGs and DGDGs from spinach leaves are hydrolyzed *in vitro* by pancreas homogenates from sheep, rats and guinea pigs to free fatty acids (FFA), monogalactosylmonoacylglycerol (MGMG), monogalactosyldiacylglycerol

(MGDG), and water-soluble galactose-containing compounds <sup>96</sup>. The higher galactolipase activity found in guinea pig pancreas can today be explained by the identification of GPLRP2 as the mammalian galactolipase with the highest activity characterized so far <sup>79</sup> (Table 4). The ability of commercial porcine pancreatin to deacylate MGDG, DGDG <sup>64-66, 69</sup> and SQDG <sup>97</sup> was also reported.

The intestinal digestion of galactolipids in humans was however largely ignored before Andersson et al. examined their digestion *in vitro* using human duodenal contents, pancreatic juice, and purified human pancreatic CEH, and colipase-dependent pancreatic lipase (Lip-Col complex) <sup>98</sup>. [<sup>3</sup>H]galactose or [<sup>3</sup>H]FA-labelled DGDG were used as substrate and incubated with these various sources of enzymes. Pancreatic juice and duodenal contents hydrolyzed DGDG to FFAs, DGMG and water-soluble galactose-containing compounds. The hydrolysis of DGDG was bile salt-dependent and was optimum at pH 6.5-7.5. Human pancreatic juice released FAs from MGDG, DGDG, and SQDG. Purified CEH was found to hydrolyze all three substrates, with a higher activity on MGDG than on SQDG and DGDG. Purified Lip-Col complex had some activity toward MGDG but had little activity against DGDG. Separation of pancreatic juice by Sephadex G100 gel filtration chromatography revealed two peaks with galactolipase activity that coincided with the elution of CEH (molecular mass around 100 kDa) and pancreatic lipase (molecular mass around 50 kDa). In contrast to Lip-Col, enzymes of the latter peak were as active against DGDG as against MGDG. Thus, it was deduced that DGDG was hydrolyzed by CEH and by a pancreatic enzyme(s) with a molecular mass similar to that of pancreatic lipase (50 kDa). This finding was made at the same time as the identification of novel members of the pancreatic lipase gene family, expressed in the pancreas and found in the exocrine pancreatic secretion : the so-called pancreatic lipase related-proteins (PLRP) 1 and 2 <sup>89, 99-101</sup>. Soon after, it was demonstrated that purified GPLRP2, sharing around 64% amino acid identities with classical

pancreatic lipase<sup>102, 103</sup>, displays a high galactolipase activity towards DGDG (250 U/mg of enzyme, with 1 U= 1  $\mu$ mole fatty acid released per minute), MGDG (160 U/ mg) and SQDG (160 U/ mg) while purified human pancreatic lipase (HPL) was poorly active (0.1-0.3 U/mg) on these substrates and CEH was around 100-fold less active than GPLRP2 under similar assay conditions<sup>104</sup>. Further characterizations of PLRP2 in rat<sup>86, 104</sup>, horse<sup>105</sup>, rabbit<sup>105</sup>, and humans<sup>67, 87, 101, 106</sup> have confirmed the galactolipase activity of PLRP2, as well as the fact that it is the most active galactolipase present in the exocrine pancreatic secretion<sup>67, 79, 104</sup> (Table 4). Recently, PLRP2 was also identified in porcine pancreatic juice<sup>107</sup>. This later study confirmed that galactolipase activity in both human and porcine pancreatic juices is associated with two protein fractions of around 100 and 50 kDa, containing CEH and PLRP2, respectively. Although human CEH has a lower specific activity than HPLRP2 (Table 4), it is present at higher levels than HPLRP2 in human pancreatic juice and duodenal contents<sup>107, 108</sup> and one can assume that it has also a significant contribution to galactolipid digestion. In some species, like Zebrafish, PLRP2 is not present while genes encoding homologs to pancreatic CEH can be found using a BLAST search (see Genebank AAH55668 and NCBI Reference Sequence NP\_001020344.2).

Most studies on the digestion of galactolipids by pancreatic enzymes have been performed with MGDG, DGDG and SQDG purified from lipid extracts of plant leaves, such as spinach<sup>67, 96</sup> and *Artemisia princeps* leaves<sup>65</sup>. Studies on galactolipid digestion by direct action of pancreatic enzymes on whole plant materials are poorly documented. Recently, Wattanakul et al.<sup>109</sup> studied the *in vitro* digestion of chloroplast-rich fractions (CRF) prepared from spinach leaves and a green residue from agriculture, pea vine haulm<sup>110</sup>. They used a two-step static digestion model including a gastric phase with rabbit gastric extracts as a source of pepsin and gastric lipase, followed by an intestinal phase with either human pancreatic juice (HPJ) or porcine pancreatic extracts (PPE or pancreatin) as sources of

pancreatic enzymes (Figure 11). No significant hydrolysis of galactolipids was observed during the gastric phase, thus confirming that gastric lipase has no galactolipase activity. MGDG and DGDG from both spinach and pea vine haulm CRFs were however largely converted into MGMG, DGMG (Figures 9A and B) and FFA (Figure 11C and D) during the intestinal phase, with conversion rates >80% for MGDG and >60% for DGDG after 60 minutes of incubation with pancreatic enzymes. It was observed that the digestion of CRF galactolipids by PPE is less effective than the digestion by HPJ, in good agreement with the fact that PPE contains lower levels of galactolipase activity compared to HPJ <sup>107</sup>. The composition of FFA was similar to the FA composition of the whole CRF, with a large proportion of ALA ( $55.38 \pm 1.33\%$  w/w of the total FA in spinach leave CRF and  $37.82 \pm 0.31\%$  w/w of the total FA in pea vine haulm CRF). Thus, CRFs from green biomass are well digested by pancreatic enzymes and represent an interesting source of ALA for human or animal diet supplementation. Spinach CRF prepared from blanched fresh leaves contain  $35.56 \pm 2.56$  mg ALA per g of DW. ALA levels are not as high in the pea vine haulm CRF ( $14.29 \pm 2.06$  mg ALA per g of DW), probably because some endogeneous hydrolysis of galactolipids and further oxidation of ALA occurs in this green waste material before it is collected and further heat-treated to inactivate endogeneous enzymes before CRF preparation <sup>109</sup>. The galactolipid composition of CRFs is given in Table 1.

#### **4. Intestinal absorption and metabolic fate of galactolipids**

In the early study of Bajwa and Sastry in 1974 <sup>96</sup>, when pancreas homogenates were used as the source of digestive enzymes, the major pathway identified for galactolipid digestion was the hydrolysis of the ester bonds to form FFA and intermediate lyso-galactolipid products, MGMG and DGMG, and finally galactose and glycerol. Anderson et al. reached the same conclusion using human duodenal contents <sup>98</sup>, and PLRP2 and CEH were

identified as the pancreatic enzymes involved in the conversion of galactolipid into FFA and lyso-galactolipids<sup>98, 104</sup>. Ohlsson et al. then used [<sup>3</sup>H]FA-labeled DGDG and [<sup>14</sup>C]-labeled DGDG mixed with soybean oil ± phosphatidylcholine (PC) orally given to rats to determine whether some galactolipids may be absorbed intact or if complete lipolysis and reesterification of the galactolipid FAs into chyle TAGs and phospholipids occur. Rats were fed by gastric intubation and some of them were killed 2 and 4 h later for blood and gastrointestinal organs collection, followed by lipid extraction. In other rats, one-day faeces were collected after oral feeding and their lipids were extracted. In other experiments, mesenteric lymph duct cannulation was performed and radioactive chyle was collected for 8 h after feeding the rats. The radioactivity present in total lipid extracts and aqueous phases, as well as in individual lipid classes separated by TLC was determined. In plasma, [<sup>3</sup>H] radioactivity was mainly found in neutral lipids (around 80 %), while in the liver, it was mainly recovered in phospholipids (around 50% and mostly in PC). The intestinal contents radioactivity was mainly found in the DGDG fraction (around 55 %) and neutral lipids (around 25 %), suggesting the occurrence of some acyl transfer between DGDG and neutral lipids. The total recovered [<sup>3</sup>H] radioactivity in chyle was 12.3 ± 2.1 % during 8 h after feeding the rats, and 90% of this radioactivity was found in neutral lipids, mainly in TAG. The polar lipids in chyle were dominated by PC, and little or no radioactivity was detected in lipid bands separated by TLC that could correspond to intact galactolipids. Liver contained the highest levels of radioactivity of all other organs investigated. No intact DGDG was found in the tissues. The radioactivity recovered in feces was mainly associated with the aqueous phase and thus with water-soluble compounds. Similar results were obtained when rats were fed with [<sup>14</sup>C]-DGDG containing emulsion, with respect to total recovered radioactivity in chyle (15.3 ± 7.6%) and distribution to neutral and polar lipids. The lymph

duct cannulation experiments indicated that no detectable galactolipids were present in chyle lipids.

In conclusion, this study <sup>111</sup> showed that the FAs present in DGDG given orally to rats are released in the gastrointestinal (GI) tract and further re-esterified in TAG and phospholipids found in chyle and plasma. There is no evidence so far that lyso-galactolipids could be absorbed in the intestine and further used for galactolipid resynthesis in the enterocyte. Nevertheless, one has to mention that these lipids are not taken into account in lipidomics studies dedicated to intestinal absorption and plasma lipids.

## **5. Relationships between the digestion of galactolipids in herbivores and the fatty acid composition of derived products (meat, milk)**

Herbivore species are those that ingest the highest amounts of galactolipids and it has been estimated that MGDG may account for as much as 60% of their dietary lipids <sup>105</sup>. For instance, guinea pigs can consume up to 700 mg galactolipids a day on average (or 0.58 to 1 g per kg of body weight per day) and humans only 200 mg a day (or 0.003 g per kg of body weight per day, for a mean body weight of 62 kg). A daily intake of 700 mg of galactolipids corresponds to approx. 390 g of fresh grass according to the galactolipid composition of red clover given in Table 1 (1795 mg/kg fresh weight) and estimated from <sup>112</sup>. The daily intake of galactolipids in guinea pig is in fact in the same order of magnitude as the daily intake of fat in humans from western countries (approx. 100 g/day; mostly TAG; 1.6 g per kg of body weight per day, for a mean body weight of 62 kg). It is therefore not surprising that in the guinea pig, the digestive galactolipase PLRP2 is produced at high levels, similar to those of classical pancreatic lipase <sup>103</sup>. Similar observations have been made in the closely related species, *Myocastor coypus* <sup>113</sup>. More generally, high levels of PLRP2 have been found in

monogastric herbivores including horse and rabbit <sup>105, 114</sup>. These findings support the association of PLRP2 with the herbivore diet and the digestion of galactolipids. Nevertheless, the active enzyme was not identified so far in the pancreas or pancreatic secretion of ruminants (cow, goat and sheep) nor in the pancreas of carnivores (dogs and cats) <sup>105</sup>. The *plrp2* or *pnplrp2* gene is however present in these species (see GenBank accession numbers BC142351 (cow), XM\_005698515 (goat), M\_012102995 (sheep), XM\_022411916 (dog) and XM\_003994441 (cat)), in which PLRP2 may have a different physiological function, such as the digestion of phospholipids <sup>89</sup> or a role in the immune system <sup>90, 115, 116</sup>. In the goat for instance, active PLRP2 was identified in the secretion of bulbo-urethral gland and in seminal plasma <sup>117</sup>.

In a genetic study on human adaptations to diet, a mutation of the *plrp2* gene was found to be strongly correlated with populations that use cereals as the main dietary component <sup>118</sup>. This finding was tentatively associated with the high galactolipid contents in cereals (Table 2) and the galactolipase activity of PLRP2 <sup>119</sup>. A stop codon is however introduced by the single-nucleotide polymorphism found in *plrp2* (SNP rs4751995; c.1074G > A,) that results in the W358X mutation and a premature truncation of HPLRP2 after residue S357. The resulting variant contains the catalytic N-terminal domain but is missing the entire C2-like C-terminal domain (Figure 6). To date, this domain has been mainly associated with colipase binding and interaction with the oil-water interface in the classical pancreatic lipase <sup>120, 121</sup> and therefore its lipase activity on TAG emulsions. In PLRP2s, most residues of the C2-like C-terminal domain involved in the interaction with colipase are mutated and interaction with colipase is weak if not null <sup>89</sup>. Moreover, several members of the pancreatic lipase gene family found in insects and showing high phospholipase activity <sup>122</sup> or presumably galactolipase activity <sup>123, 124</sup> do not have the C2-like C-terminal domain (Figure 6). It was therefore tempting to associate the PLRP2 mutation found in humans with an adaptation to a



specialized diet and a more active version of the enzyme towards galactolipids <sup>119</sup>. However, Xiao et al. <sup>125</sup> did not succeed in producing and characterizing the corresponding PLRP2 variant (W340X according to their different amino acid numbering that does not take into account the signal peptide) using the *Pichia pastoris* yeast heterologous expression system or transfected COS7 and HEK 293T cells. The protein was poorly secreted and largely retained inside the cell, probably due to protein misfolding. We also failed to produce this HPLRP2 variant in the yeast, as well as all PLRPs missing the C2-like C-terminal domain (unpublished data from Frédéric Carrière). Therefore, it is still unknown whether the HPLRP2 variant may display galactolipase activity.

Perhaps the best demonstration of galactolipid digestion by endogeneous enzymes in monogastric herbivore species and subsequent intestinal absorption of their PUFA comes from the characterization of horse-meat FAs. The FA profile of horse-meat has distinct features compared to bovine and other more common ruminant meats. The horse has the ability to efficiently transfer PUFA from the diet into meat <sup>126</sup>. It is a hindgut fermentation herbivore with a relatively small stomach, adapted to continuous eating, mainly free-ranging in grassland environments. The digesta passage rate through the horse stomach and small intestine is quite fast in comparison to ruminants <sup>127</sup>, reaching the hindgut fermentative compartments 3 h after feed consumption <sup>128</sup>. Due to the post-gastric localization of digestive fermentative chambers in horses, and contrary to polygastric herbivores, dietary FAs are released and absorbed before being submitted to extensive microbial metabolism. This allows an efficient absorption and deposition of PUFAs from pasture species into tissues before the PUFAs are subjected to microbial biohydrogenation in the hindgut. It is therefore assumed that horses have a high ability to hydrolyse galactolipids from grass in their upper GI tract. Since it is now established that horses produce high amounts of PLRP2 <sup>105, 114</sup>, the digestion of galactolipids by horse PLRP2 could potentially explain the high content of ALA deposition

in horse tissues<sup>129-131</sup>. This accumulation of ALA was particularly evidenced when horses were fed under extensive conditions (grass feeding) compared to intensive conditions (animals fed with concentrates) before slaughter (Table 5)<sup>132, 133</sup>. Indeed, ALA could account for as much as 24.3 % of total FAs in subcutaneous adipose tissues of horse-meat when horses had free-ranging grass feeding. These data indicate that the horse might be one of the best species to transfer n-3 PUFAs from pastures to humans as suggested by Guil-Guerrero et al.<sup>134</sup>.

The variability in PUFA percentage in horse-meat is however quite significant and ranges from 15.6% in 3-year-old horses fed commercial concentrates<sup>129</sup> to 46 % in 2-year-old grass-fed animals<sup>131, 133</sup>. High LA and ALA contents were observed in horse fat<sup>126, 135</sup>. It results from these high ALA levels that almost 5% of horse loins surveyed in northern Spain contain over 300 mg of ALA per 100g of meat which could likely be marketed as a "source" of n-3 FAs according to European Union Regulation No 116/2010<sup>136</sup>. In horses, intramuscular ALA is preferentially deposited in the neutral lipid fraction, while LA and n-3 and n-6 VLC-PUFAs are incorporated into the polar lipids<sup>137</sup>. These findings support the previous observation made by Ohlsson et al. in rats that ALA ingested via the intake of galactolipids and absorbed at the intestinal levels is preferentially re-incorporated in TAG<sup>138</sup>. Another strong finding of the study by Belauzaran et al. is the identification of hexatrienoic acid (16:3 n-3) in the neutral lipids from horse muscle, with higher levels (0.072 mg/100 g of fresh meat) in suckling foals produced under grazing conditions than in another group of foals finished with concentrate (0.010 mg/100 g of fresh meat)<sup>137</sup>. The presence of 16:3 n-3 (plastidal prokaryotic pathway of FA biosynthesis) in horse meat is a specific marker of the transfer of the PUFA from grass galactolipids into the animal tissues.

Still concerning horses, a remarkable finding was made in the fat of a frozen horse found in the permafrost of Siberia. The high amount of ALA detected (4.2 % of total FA,

which may correspond to an initial amount of around 20 %) had never been detected in any sample from frozen prehistoric animals or humans. This finding suggests a high intake of ALA by horses of the Upper Palaeolithic and Neolithic, which could have contributed to fulfil the daily needs of n-3 FAs for hunters at the time in which these animals lived <sup>134</sup>.

In ruminant herbivores like cow, goat and sheep, PUFAs released from grass galactolipids cannot be absorbed as such, because of the bacterial biohydrogenation of PUFA occurring in the rumen that converts PUFA into SFA <sup>139</sup>. It is assumed that prior to their hydrogenation, PUFAs can be released from galactolipids by the combined action of endogeneous galactolipases present in grass and galactolipases produced by the rumen microbiota <sup>82</sup>. An indirect impact of the biohydrogenation of PUFAs in ruminants is that the n-3 PUFAs are found at very low levels in their derived products such as meat and milk. An interesting finding made with mares's milk from different breeds is that, like horse meat, it contains high levels of ALA (up to 24 % of total milk FA) <sup>140</sup>. ALA represents for instance  $20.28 \pm 10.15$  % of total milk FA in Wielkopolski Horse,  $13.35 \pm 6.15$  % in Konik Polski, and  $10.20 \pm 2.51$ % in Polish Cold-blooded Horse <sup>141</sup>. In donkeys, reared under semi-extensive conditions, in which we assume that galactolipid digestion is similar to that in horse, ALA levels in milk can reach 11 % of total FAs after 7 months of lactation <sup>142</sup>. In other monogastric herbivores like guinea pigs and rabbits, ALA levels in milk can reach 5.5 and 1.7% of total FAs, respectively, which is still higher than in cows's milk <sup>143</sup>. Like in horse, these high ALA levels in milk are correlated with high ALA levels in the adipose tissue (5 and 1.7%, respectively, in guinea pigs and rabbits) <sup>143</sup>. Therefore, one can conclude that the mode of digestion of grass galactolipids in herbivores has a major impact on the FA composition of both meat and milk.

## 7. The digestion of galactolipids in fish

Another demonstration of the absorption and further distribution in tissues of the PUFAs from galactolipids was made recently in a study on the digestion of plant chloroplasts incorporated into fish diets<sup>144</sup>. In this study, zebrafish (*Danio rerio*) were fed during an eight week time period either with a standard aquaculture fishmeal or with diets in which part of the fishmeal (10, 20 or 50% w/w) was replaced by either a whole spinach leaf powder (SLP) or a chloroplast rich fraction (CRF) prepared from spinach leaves according to<sup>145</sup>. At the end of the trial the fish were euthanized, weighed and their total lipid were extracted before FA derivatization and analysis by GC-MS. While oleic and LA were found in higher proportions in the fish fed with the control fish diet, palmitic acid (C16:0), hexadecatrienoic acid (C16:3 n-3) and ALA, which are characteristic of galactolipids, were all significantly higher in the zebrafish fed with the diets containing CRF and SLP compared to the controls (Table 6). As seen previously in horses, the presence of C16:3 in the zebrafish tissues is a unique marker of the reincorporation of galactolipid FAs in the endogeneous fish lipids. Thus, this study clearly shows that the galactolipids from CRF and SLP are digested by zebrafish, releasing PUFA that are taken up by the fish<sup>144</sup>. In addition to the uptake of ALA, its into VLC-PUFAs (EPA and DHA) was also observed.

We searched for orthologs of genes encoding mammalian digestive lipases in the zebrafish genome (*Danio rerio*, assembly GRCz11) to identify the enzymes possibly involved in the digestion of galactolipids. A BLAST search did not locate any ortholog of the classical pancreatic lipase (*pnlip*), colipase (*clps*) or pancreatic lipase-related proteins 1 (*pnliprp1*) and 2 (*pnliprp2*) in zebrafish. A tandem duplicate of genes encoding orthologs to pancreatic CEH was however identified (Genebank AAH55668 for tandem duplicate 1; NCBI Reference Sequence NP\_001020344.2 for tandem duplicate 2) and the corresponding proteins named DrCEH1 and DrCEH2 (Figure 10). With 531 amino acid residues in the predicted mature

proteins, these two zebrafish CEH are shorter than human pancreatic CEH (733 amino acid residues; UniProtKB/Swiss-Prot: P19835.3). This is due to the absence of a large C-terminal region made of proline-rich tandem repeats of 11 amino acids (Figure 10), with 17 repeats in human CEH (some variations between individuals can be observed) and various numbers of repeats in other mammalian species (4 in porcine CEH <sup>107</sup>). The absence of the C-terminal region was previously reported for the CEH-BSSL of another fish species, the salmon <sup>146</sup>. In their conserved parts, Zebrafish and human CEHs share 58 % amino acid identity, while two zebrafish CEHs share 87 % amino acid identity. We built 3D models of DrCEH1 and DrCEH2 based on the known X-ray structure of the truncated human CEH (PDB: 1JMY) missing the C-terminal domain <sup>147</sup>) and we confirmed that a DGDG molecule can be docked in a cavity similar to that found in human and bovine CEH (Figure 9). To our knowledge, the zebrafish CEHs have not been isolated and biochemically characterized so far and their putative galactolipase activity remains to be shown. Nevertheless, non-specific bile-salt activated lipase activity (presumably that of CEH) in the gut of *Danio rerio* was measured using p-nitrophenyl myristate as substrate in the presence of sodium cholate <sup>148</sup>. The zebrafish fed with a herbivorous diet had significantly higher lipase activities in comparison to fish fed with carnivorous ( $\approx 3$ -fold) and omnivorous ( $\approx 1.5$ -fold) diets, which supports a correlation between the secretion of CEH (named carboxylester lipase, CEL, in this study) and the digestion of plant lipids.

A BLAST search for CEH ortholog in a typical fish herbivore, the grass carp *Ctenopharyngodon idella*, also reveals the presence of one CEH (GenBank: ACV04933.1), while genes for pancreatic lipase, pancreatic-lipase proteins 1 and 2 and colipase are absent like in zebrafish. The grass carp is particularly interesting because it exhibits food habit transition from carnivory to herbivory during development. He et al. performed a transcriptome analysis of the grass carp <sup>149</sup> and they found that the expression of genes

involved in digestion and metabolism was significantly different between fish before and after food habit transition (from feeding with chironomid larvae (*Chironomus tentans*) to feeding with duckweed (*Lemna minor*)). The mRNA expression levels of several pancreatic enzymes including trypsin, pancreatic elastase, carboxypeptidase A, carboxypeptidase B, bile salt-stimulated lipase/ carboxyl ester hydrolase (CEH) and secretory phospholipase A2 (PLA2) were significantly higher in the fish fed with duckweed. Interestingly, fish fed with this low nutritional plant-based diet had higher growth than those fed with high nutritional animal diets, which suggests that herbivorous fish species have evolved the ability to access to and to concentrate the energy stored in the nutrients spread in the plant biomass by producing more digestive enzymes and by consuming more food per day. The grass carp and herbivorous fish in general <sup>150, 151</sup> has a much longer gut than carnivorous and omnivorous fish, what can favour the contact time between the digestive enzymes and the plant biomass, as well as the intestinal absorption processes. The expression of several genes involved in the production of bile is also increased in the grass carp. Thus, herbivorous fish could use these adaptations and the production of CEH to release and absorb the FAs from galactolipids, the main acylglycerolipids present in their diet.

It was observed that CEH (or CEL) activity is elevated in fishes consuming low-lipid and high-fibre foods <sup>148, 152, 153</sup>. From the genome sequencing of the herbivorous prickleback fish (*Cebidichthys violaceus*), extensive genetic variations and adaptive amino acid variation for CEH were revealed, suggesting that CEH is associated with the dietary adaptation of *C. violaceus* physiology <sup>154</sup>. Four tandem copies of CEH (cel-1a, cel-1b, cel-1c and cel-2) and one CEH-like (cel-like) locus were identified in the *C. violaceus* genome. cel-2 and cel-like are highly expressed in the liver and proximal intestine, while cel-1a, b and c are expressed at a lower level and mainly in the proximal intestine. Thus, it appears that herbivorous pricklebacks, and other herbivorous fishes invest in the production of lipases to ensure the

digestion of lipids present in their algal diet. Since algae mostly contain galactolipids in their membranes, Heras et al. also came to the conclusion that CEH (or CEL) could be the main fish lipase involved in the digestion of galactolipids <sup>154</sup>. According to these authors, the production of numerous lipases is consistent with the nutrient balancing hypothesis, under which animals invest in the synthesis of digestive enzymes to acquire limiting nutrients <sup>155</sup>. Since galactolipids are the most abundant acylglycerolipids, we would use the term “dispersed” rather than “limited” to qualify their lower availability. This dispersed state of galactolipids in the biomass membranes first and in the micellar phase of the GI contents second, may require more enzymes to achieve an efficient digestion compared to the digestion of TAG by lipases. Indeed, the later act at a very high rate on the TAG molecules concentrated in lipid droplets while lipolytic enzymes acting on water-dispersed substrates usually display a lower turnover <sup>156</sup>. Since there is strong interest in using more plant-based feeds for aquaculture, like the CRF previously mentioned <sup>144</sup>, a better knowledge on the adaptation of fish to an herbivorous diet via the production of galactolipases may be helpful for species selection as well as for the generation of genetically modified aquaculture fishes <sup>154</sup>.

Apart from the findings in zebrafish, we searched for other correlations between the digestion of galactolipids and FA composition of fishes. ALA was found to be the predominant n-3 PUFA in several farmed fishes with the highest levels ( $7.91 \pm 3.60$  % of total FA) observed in the herbivorous grass carp (Table 7) <sup>157</sup>. High levels of ALA ( $\geq 5$  % of total FA) are also found in wild freshwater fish with planktivorous (bleak, shad, whitefish) and omnivorous (rudd) dietary habits <sup>158</sup>.

## 8. The digestion of galactolipids by foliovorous insects

Lepidopteran larvae have an essential requirement for the long-chain PUFA, LA and ALA<sup>159</sup> and their absence leads to failure in development and wing deformities in adults<sup>160</sup>. These PUFA must be obtained from the diet, using lipases secreted into the midgut lumen. EST libraries have been used to identify the lipases expressed in the midgut of the light brown apple moth, *Epiphyas postvittana*<sup>161, 162</sup> and the polyphagous caterpillar pests, *Helicoverpa armigera* and *Helicoverpa zea*<sup>163</sup>. Search for lipase genes has also been performed in the genomes of other species representing the four largest orders of holometabolous insects, the fruit fly *Drosophila melanogaster* and the malarial mosquito *Anopheles gambiae* (Diptera), the silkworm *Bombyx mori* (Lepidoptera), the honey bee *Apis mellifera* (Hymenoptera) and the flour beetle *Tribolium castaneum* (Coleoptera)<sup>123</sup>. Several proteins with sequence identity to mammalian pancreatic (neutral) and gastric (acid) lipases have been found. The insect lipases from the acid lipase gene family are however expressed at very low levels relative to those of the pancreatic lipase gene family<sup>123, 162</sup>. Since no member of the acid lipase gene family has been found to display galactolipase activity so far, focus has been made on the pancreatic lipase gene family members among which insect galactolipases could be found. These previous studies have however ignored the possible presence of CEH- or BSSL-related lipases that belong to the acetylcholine esterase-like family of lipases. A BLAST search for homologs of human pancreatic CEH in the insect genomes mentioned above reveals however the presence of several genes annotated as “bile-salt activated lipase-like” (NCBI reference sequences XP\_021192219.1, XP\_021192218.1, XP\_021192217.1, XP\_021192221.1 and XP\_021196777.1 in *Helicoverpa armigera* (also known as HaOG200133, HaOG200134, HaOG200177, HaOG200178 and HaOG200156, and corresponding to HaCCE011a, HaCCE011b, HaCCE011c, HaCCE011d and HaCCE006f in<sup>163</sup>; XP\_004930124.1 and XP\_021204925.1 in *Bombyx mori*; XP\_026299635.1 in *Apis mellifera*) and several genes with



similar levels of homology/identity (around 30%) often annotated carboxylesterase or acetylcholine esterase-like and classified among carboxyl/cholinesterases (CCEs) in <sup>163</sup>. Several genes coding for these CEH-like enzymes, including HaCCE011a, HaCCE011c and HaCCE006f, are expressed at various levels in *Helicoverpa* midgut <sup>163</sup>. As observed in fish, the insect CEHs are missing the C-terminal region with tandem repeats of proline-rich peptides found in mammalian CEH. Therefore, with several PL- and CEH-related enzymes, insects possess a very large number of potential candidates for galactolipase activity.

Expressed sequence tags from the midgut of the foliovore *Epiphyas postvittana* have shown the expression of 10 lipase genes related to pancreatic lipase (EpLIP) out of the 12 EpLIP found in the complete genome <sup>161</sup>. The two others (EpLIP7 and EpLIP9) are present only in an antennal EST library <sup>164</sup>. Only 4 of the midgut EpLIPs (EpLIP1, EpLIP 2, EpLIP 3, EpLIP12) are supposed to code for active enzymes with the conservation of the three amino acids (Ser-Asp/Glu -His) forming the catalytic triad in pancreatic lipase. In the 6 others (EpLIP4, EpLIP 5, EpLIP 6, EpLIP8, EpLIP10 and EpLIP11), the active site serine is replaced by either a glycine or a glutamic acid residue. So far, the function of these inactive proteins has not been identified, but they might be accessory proteins involved in the binding and transport of dietary lipids and lipolysis products. The midgut expression of EpLIP1 and EpLIP2 were strongly increased (~100-200-fold and 50-fold, respectively) and those of EpLIP3 and EpLIP12 moderately increased (3 to 4-fold) when larvae were fed with apple leaves compared to the artificial control diet containing cellulose, casein and wheat germ, which suggests that these four enzymes could be involved in the digestion of galactolipids in *Epiphyas postvittana* <sup>162</sup>. In the genome of *Helicoverpa armigera*, several expanded clusters of genes coding PLRPs have been identified, the largest three containing 13, 7 and 5 genes, respectively, for a total of 61 pancreatic/neutral lipase-related genes <sup>163</sup>. Many of these lipases show large deletions within the lid region but some other PLRPs have large lids. As observed

in *Epiphyas postvittana*, six are predicted to be inactive with the active site serine replaced by either a glycine in 5 of them and the catalytic triad histidine replaced by a glycine in one of them. Fifty of them possess predicted signal peptides and could be secreted extracellularly. Several of these genes show a high expression in the foregut and midgut (HarmLipases 42, 43, 67, 66, 69, 70, 71 72, 80, 82, 84, 85 and 89) and the expression of the diet-responsive lipases was most often upregulated when larvae were grown on host plants (tobacco, *Arabidopsis* and green bean) as opposed to the laboratory diet that contained higher levels of FFAs than the host plants.

The midgut-expressed members of the pancreatic lipase gene family constitute a new grouping within the pancreatic lipase superfamily, all of them sharing the striking deletion of the C-terminal C2-like domain found in most members of this lipase gene family, except vespid phospholipase A1<sup>83, 122</sup> and a pancreatic lipase-related protein (PY-PLRP; NCBI Reference Sequence: XP\_021359581.1) found in the yesso scallop *Patinopecten yessoensis* (or *Mizuhopecten yessoensis*)<sup>165</sup>. Within the remaining N-terminal catalytic domain, there is a structural similarity with the guinea pig PLRP2 (Figure 6), phosphatidyl serine-specific phospholipase A1 (PS-PLA1) and vespid PLA1 in that they show a large deletion within the lid region controlling the access to the enzyme active site<sup>83, 85</sup>. Since the similarity with PLRP2 suggested the presence of galactolipase activity in the lepidopteran midgut, biochemical studies were undertaken to confirm this hypothesis. Galactolipase, phospholipase and lipase activities in the larval midgut of six species of lepidopteran adapted to four distinct types of diet and the response to changes in the lipid composition of the diet of these insects were measured<sup>124</sup>. The insects chosen for this study were two foliovores, the Lightbrown Apple Moth *Epiphyas postvittana* (Tortricidae) and the Cotton Bollworm *Helicoverpa armigera* (Noctuidae), two granivores, the Indian Meal Moth *Plodia interpunctella* (Pyralidae) and the closely related Mediterranean Flour Moth *Ephesia kuehniella* (Pyralidae), a presumptive

carnivore, the Greater Wax Moth *Galleria mellonella* (Pyralidae), and a keratinophage, the Common Clothes Moth *Tineola bisselliella* (Tineidae).

All insects were grown on their natural diets but additionally several species were also grown on artificial diets designed for optimal growth, survival and normal development. Galactolipase, phospholipase and lipase activities with alkaline optimum pH of 9 to 10.5 were detected in the midgut of all six species tested. However, there was a clear difference between the levels of the different lipolytic activities in the two foliovore species (*E. postvittana* and *H. armigera*) compared to the four non-foliovore species (Figure 12A). The average level of galactolipase activity in foliovores was 6.7-fold higher than in non-foliovores, the average level of phospholipase activity was 5.9-fold higher whereas the average level of lipase activity was 1.7-fold lower. This is reflected in the ratios of galactolipase to lipase activities ranging from 3 to 9 in the phytophages while they are <0.7 in the non-phytophages (Figure 12B).

For the foliovore *E. postvittana*, switching from a galactolipid and phospholipid-rich diet (apple leaves) to a TAG-based artificial diet (wheatgerm) induced a 3.3-fold decrease in the midgut galactolipase activity, while phospholipase and lipase activities were not changed significantly. Switching *E. postvittana* feeding from wheatgerm to a fat-free artificial diet led to a lipolytic activity pattern similar to that of the apple leaf-feeding larvae, with a 3-fold increase in galactolipase activity. In the second foliovore *H. armigera*, all lipolytic activities were found at very low levels during starvation and increased when the larvae were fed on tomato leaves or limabean diets, with galactolipase activity being the highest one<sup>124</sup>.

Conversely, the lipolytic activities in the four non-foliovore species were dominated by high levels of lipase activity, including in the two granivorous species, *P. interpunctella* and *E. kuehniella*, grown on cereal grain-based diets. These diets contained however higher levels of TAG than galactolipids<sup>124</sup> Interestingly, galactolipase activity was increased in *Galleria mellonella* when it was fed with honeycomb diet what could be related to the

presence of pollen, known to be rich in galactolipids<sup>166</sup>. Thus, this carnivorous insect that can usually obtain dietary lipids from the TAG stored in bee larvae and pupae may also use galactolipids as a source of FAs.

From these findings, the lepidopteran larval adaptation to lipid diet can be divided into two main groups. The first group, and dominant among lepidoptera in terms of species, is phyllophagous, eating a diet composed largely of leaves, like the two foliovores *E. postvittana*, and *H. armigera*. It is likely that these species utilise galactolipids, not only as resource for the essential PUFA but also as a rich and abundant source of energy for growth. Moths that live by eating leaves at the larval stage represent the majority of the Lepidoptera. The second group of insects, the non-foliovores, are characterised by very low levels of galactolipase and phospholipase activities and moderate levels of lipase activity. This certainly correlates with the presence of higher TAG levels in the normal diet of the two granivorous (*P. interpunctella* and *E. kuehniella*) and carnivorous (*G. mellonella*) species.

The lipolytic activity changes with diet observed for *E. postvittana* larvae by Christeller et al.<sup>124</sup> also correlate with mRNA levels in the midgut of larvae fed a fat-free diet which are very similar to those of larvae fed apple leaves<sup>162</sup>.

We tried to isolate and identify the enzymes displaying galactolipase activity in the midgut of *Helicoverpa armigera* larvae fed on tomato leaves. The proteins present in the extract solution were separated by anion exchange chromatography (monoQ HR5/5) using a NaCl concentration gradient and the fractions obtained showing galactolipase activity were subjected to SDS-PAGE. The main protein bands were excised from the gel, digested by trypsin and analyzed by mass spectrometry. From a protein band of around 40 kDa, peptides corresponding to one *Helicoverpa armigera* PLRP (HaPLRP; neutral lipase 74 (OGS# 200601) according to<sup>163</sup>; NCBI Reference Sequence: XP\_021199604.1) could be identified with a low sequence coverage of 16 % (Figure 6). HaPLRP was predicted to be secreted with

a signal peptide of 16 amino acid residues and a mature polypeptide of 318 amino acids and a molecular mass of 34.576 kDa. It shares 32.27 % and 30.9 % amino acid identities with HPLRP2 and GPLRP2, respectively (Figure 6). It has no C-terminal C2-like domain and like GPLRP2, it has a large deletion within the lid region. A 3D homology model of HaPLRP was built that shows a free access to the enzyme active site (Figure 7D). A DGDG molecule could be docked in the active site cavity whose topology is similar to those observed in HPLRP2 (Figure 7A) and GPLRP2 (Figure 7B). Therefore, we assume that HaPLRP could be one of the enzymes displaying galactolipase activity in the midgut of *Helicoverpa armigera*.

A question remains concerning the presentation of galactolipids to lipolytic enzymes in the insect midgut. Indeed, insects do not have bile secretion and do not produce bile salts, while mixed galactolipid-bile salt micelles as substrate were used to measure galactolipase activities in midguts<sup>124</sup> and assay conditions optimized for mammalian galactolipases<sup>88</sup>. A search for other surfactants present in the insect secretions that could play a role similar to that of bile salts in the midgut identified the fatty acid–amino acid conjugate (FAC) volicitin (N-(17-Hydroxylinolenoyl)-L-glutamine) and its analogues as potential candidates. We tested the effects of the volicitin analog N-linolenyl-L-glutamate (NLLG) on the galactolipase and phospholipase activities of *Helicoverpa armigera* midgut (Figure 13) using NLLG concentrations close to those estimated for FACs in the *Spodoptera* midgut (~0.7 mM as estimated from<sup>167</sup>). It was found that the presence of NLLG allowed measuring significant galactolipase and phospholipase activities and at higher concentrations, NLLG was equally effective as sodium taurodeoxycholate. These findings (Christeller JT, unpublished data) suggest that, FACs have the potential to form mixed micelles with galactolipids in the insect midgut and thus trigger their digestion by galactolipases.

Another finding supporting the importance of galactolipid digestion in insects maybe the high levels of ALA found in honeycomb (up to 36 mg/kg fresh weight; 250 mg/kg of total

lipids)<sup>168</sup> and bee bread<sup>169</sup>, in which ALA is the main FA. Since galactolipids and phospholipids are the major acylglycerolipids and ALA is the major FA present in pollen<sup>166</sup> and since bee bread is processed pollen, mixed with digestive secretions and further stored in the honeycomb cells by the bee, one can assume that bees have the ability to efficiently digest pollen galactolipids and phospholipids. A BLAST search reveals the presence of many pancreatic lipase-related proteins (at least 39 NCBI sequences) and one CEH homolog (NCBI reference sequence XP\_026299635.1) in the honey bee, *Apis mellifera*.

We searched for a correlation between the digestion of galactolipids and FA composition of insects. A high proportion of ALA is characteristic of Lepidoptera (22 % of total FA on average) followed by Hymenoptera (15.5 % of total FA on average)<sup>170 171</sup>. The silkworm grown on mulberry leaves was shown to contain ALA as the main FA at various stages of its development, with 35 %, 31.2-44.7 %, 30.9-41.1 % and 29.2-34.9 % of total FA in eggs, larvae, pupae and adult, respectively<sup>172</sup> (N.B. the abstract of this article wrongly mentions LA as the predominant FA). In silkworm larvae, the highest level of ALA was found in the intestine (45.9 %), followed by the blood (43.1 %), the silk gland (39.5 %) and the fat body (25.9 %)<sup>172</sup>. The FA analysis of total lipids extracted from silkworm pupae showed that ALA was the predominant FA in TAG (41.8-42.7 %), PC (36.3-38.5 %) and phosphatidylethanolamine (PE; 33.7-41.3 %)<sup>173</sup>. They have also suggested that ALA-rich oil extracted from silkworm larvae could be a valuable dietary supplement<sup>173</sup>.

## **9. The digestion of galactolipids by microorganisms**

In ruminants, it has been shown that microbial enzymes, produced by the microbiota from the rumen, are involved in the digestion of grass galactolipids<sup>82</sup>. The PUFA released under these conditions are further biohydrogenated into SFA by microbial enzymes also present in the rumen<sup>139</sup>. The biohydrogenation of ALA only occurs after it is released from galactolipids<sup>174</sup>. In dairy cows, around 85 to 100 % of the ingested ALA is biohydrogenated

in the rumen and stearic acid (18:0) is the major FA entering the duodenum<sup>175</sup>. So far, much attention has been paid to the identification of microorganisms and enzymes involved in the degradation of plant cell walls and fibers (cellulases, xylanases,  $\beta$ -glucanases, pectinases)<sup>176</sup> and FA biohydrogenation in the rumen<sup>175, 177</sup>, but the digestion of galactolipids by the rumen microorganisms has been rarely explored<sup>82, 174, 178</sup>. Using Naphtyl and p-nitrophenyl esters of FAs, esterase activities were detected in various bacteria isolated from the rumen, including *Butyrivibrio fibrisolvens*, *Bacteroides ruminicola*, *Selenomonas ruminantium*, *Ruminobacter amylophilus*, *Streptococcus bovis*<sup>179</sup>, but it was not shown whether these strains could also hydrolyze acylglycerols. Hazlewood and Dawson isolated from the sheep rumen a FA-requiring *Butyrivibrio species* (strain S2) that deacylates plant galactolipids, phospholipids and sulpholipids to obtain sufficient FAs for growth<sup>180</sup>. Phospholipase and galactolipase activities were however found in a subcellular fraction which contained fragments of plasma membrane<sup>181</sup>. Later, Lanz and Williams isolated an esterase with a molecular mass of about 66,000 Da, that was inhibited by paraoxon and diisopropyl fluorophosphate, indicating that it was a serine enzyme<sup>182</sup>. We did not find a continuation of this work, but a BLAST search for homologs to known galactolipases in the genome of *Butyrivibrio fibrisolvens* reveals the presence of a several carboxylesterases that shares 28 to 32 % amino acid identities with human CEH/BSSL and that could be potential candidates for the galactolipase activity of this rumen bacterium. In the genome of another rumen bacterium well known for its lipase activity, *Anaerovibrio lipolytica*<sup>183</sup>, no homologs to CEH are present, but we found some homology to fungal lipases (lipase 3 family) showing galactolipase activity. For instance, a lipase from *Anaerovibrio lipolyticus* DSM 3074 (GenBank: SHJ07733.1) shows 44.7 % of amino acid identities with a peptide stretch of *Thermomyces lanuginosus* lipase including the active site serine and covering 16% of the complete sequence. With the tremendous efforts made for characterizing gut microbiota, many genomes are today available for searching

putative galactolipases. Nevertheless, biochemical data on the galactolipase activity of rumen microorganisms are still missing.

The lipase 3 family of fungal lipases includes several enzymes with galactolipase activity, like the lipases from *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei*<sup>79</sup>, well known for their industrial applications and marketed by Novozymes A/S under the trade names of Lipolase™ and Lipozyme™, respectively, the lipase from *Rhizopus arrhizus* (or *Rhizopus oryzae*)<sup>63, 79</sup>, a lipase from *Fusarium solani* (FSL)<sup>80</sup> and the lipase from *Talaromyces thermophilus* (TTL) that displays the highest galactolipase activity measured so far<sup>81</sup> (Table 4). Little is known however about the physiological function of the galactolipase activity displayed by these enzymes. Their function has often been associated with their lipase activity and the uptake of FA as carbon source. Usually, the expression of fungal lipases is induced by FFA and olive oil from which FFA can be released<sup>184</sup>. TTL has the particularity to be expressed at a higher level when *Talaromyces thermophilus* is grown with wheat bran as carbon source instead of olive oil<sup>185</sup>. Wheat bran is a hemicellulosic agro-industrial residue that contains around 10 μmoles/g of total lipids, including 5 mol% of DGDG, <1 mol% of MGDG, <1 mol% of TAG, 10 mol% lysophosphatidylcholine, 2 mol% of minor polar lipids and around 75 mol% FFA<sup>186</sup>. Therefore, the induction of TTL expression by wheat bran could be due to FFA but also to the hydrolysis of DGDG and MGDG. Since *Talaromyces* species and related filamentous fungi are often isolated from cultivated soils, decaying plants and plants like guayule (*Parthenium argentatum*)<sup>187</sup>, these fungi may produce galactolipases for the uptake of FAs from the galactolipids found in their environment. *Thermomyces lanuginosus*, commonly found on leaves in compost heaps<sup>188</sup>, belongs to the same clade as *Talaromyces thermophilus* and they share similar characters, including their ability to grow at high temperatures and the secretion of a lipolytic enzymes with 90 % amino acid identity (Figure 14), showing lipase, phospholipase A1 and galactolipase activities<sup>79, 81, 185</sup>. Both fungi



belong to the same genus (*Thermomyces*) of hemicellulose degraders and therefore a galactolipase activity could complete their enzyme arsenal for the degradation of plant tissues.

In the plant pathogenic fungus *Fusarium solani*, that causes serious plant diseases throughout the world <sup>189</sup>, at least two enzymes with galactolipase activity have been identified. The first one is *Fusarium solani* cutinase (Table 4 and Figure 7C; <sup>79</sup>), already known to be a virulence factor in connection with its activity on cutine and the degradation of the lipid barrier of plant tissues. <sup>190-192</sup>. The second one is the extracellular lipase FSL (NCBI Reference Sequence: XP\_003050606.1) homologous to TTL and TLL, and showing 66 % amino acid identities with an extracellular lipase from *Fusarium graminearum* (FGL1; GenBank: AAQ23181.1) also known as *Gibberella zeae* extracellular lipase GZEL; PDB code: 3NGM) <sup>80</sup> (Figure 14). FSL could be the non-specific esterase of *Fusarium solani* involved in plant cutine hydrolysis, partly purified and described by Purdy and Kolattukudy in 1975 <sup>190, 191</sup>. Interestingly, the FGL1/GZEL homolog of FSL was found to be expressed during wheat spike infection by *Fusarium graminearum* <sup>193</sup>, simultaneously with the detection of high levels of LA and ALA in wheat spikelets, what was not observed with a FGL1-deficient mutant of *F. graminearum* <sup>194</sup>. The production of PUFA by the fungus inhibits the biosynthesis of callose, a (1,3)- $\beta$ -glucan polymer involved in plant innate immunity that acts as a physical barrier to fungi penetration by strengthening the plant cell wall <sup>194</sup>. The lipids from which the PUFA were released were however not identified in these previous studies. The observed infection process and the physiology of the infected plant organs suggested however a plant lipid as the source for PUFA <sup>194</sup>. Since ALA is mainly present in galactolipids, we hypothesize that the plant defence suppression through FGL1-dependent release of PUFA may result from the galactolipase activity of FGL1. Thus, the fungal lipases with galactolipase activity would be virulence factors like cutinases. These latter were assumed to be to exclusively required for cuticular penetration by plant pathogenic fungi,

based on the application of serine esterase inhibitors (eblactones and other organophosphorus pesticides) that could inhibit the cutinolytic activity and prevent infection of host plants<sup>192, 195-198</sup>. However, disruption of cutinase genes in *Nectria haematococca* (the asexual form of *Fusarium solani*) and other fungal pathogens did not affect their ability to penetrate the host cuticle<sup>199-201</sup>. These results indicate that cutinase inhibition is not entirely responsible for reduced virulence of plant pathogenic fungi. Therefore, other targets of serine esterase inhibitors could be virulence factors and a possible involvement of fungal lipases in the infection process was proposed<sup>193</sup>. All these findings support a role in virulence of the fungal lipases with galactolipase activity that can both disrupt plant membranes and release PUFA that favour the penetration of plant tissues by fungi. With that respect, it is worth highlighting the antagonist effects of ALA, which on one hand counteracts plant immunity and on the other hand, contributes to plant defence as a precursor of jasmonic acid<sup>202</sup>.

Molecular docking of MGDG and DGDG molecules in the active site of fungal lipases from the lipase 3 family have been reported<sup>79, 80</sup>. Based on sequence homology (Figure 14) and the known 3D structures of TLL<sup>203, 204</sup> and GZEL/FGL1<sup>205</sup>, a 3D homology model of FSL was built, in which the open conformation of the lid allows the docking of MGDG and DGDG molecules in the active site (Figure 15). The lid opening creates a large cavity in which the polar head of both MGDG and DGDG can be fitted. With respect to the  $\alpha/\beta$  hydrolase fold backbone, the topologies of the active site and the galactolipid molecules are similar to those observed in PLRPs, cutinase and CEH (Figures 7 and 9).

## **10 Galactolipids in food processing and novel foods**

In baking processes, galactolipids have an important contribution to dough properties, baking performance and resistance to bread staleness. There is a general agreement on the

improving effects of DGDG, the main galactolipid in flour (Table 2)<sup>48</sup>. However, lipolysis products like DGMG and MGMG, naturally present as minor compounds in flour seem to be the most effective compounds. Several patents related to the use of microbial galactolipases in baking processes have already been granted to companies like Novozymes A/S and Danisco A/S (see for instance patent WO98/45453). Indeed, enzymes with galactolipase activity on the DGDG found in wheat flour can release tensioactive lipolysis products that have a higher impact on the preparation of the dough.

Chu et al. have investigated the possible replacement of lecithin by galactolipids as emulsifiers and how the presence of galactolipids at the oil-water or air-water interface can impact the adsorption and activity of porcine pancreatic lipase<sup>206, 207</sup>. They studied the effects of MGDG and DGDG on the in vitro digestibility of olive oil under simulated duodenal conditions in the presence of lecithin and bile salts. It was found that olive oil lipolysis started after a longer lag phase and with a decreased lipolysis rate when emulsions were prepared with DGDG instead of MGDG. These effects were amplified by increasing the DGDG to lecithin molar ratio. It was postulated that the larger headgroup and more tightly packed molecular organization of DGDG at the interface gives rise to a steric hindrance that impairs colipase and lipase adsorption at the oil-water interface and thus delayed and reduced lipolysis. It was also observed that bile salts were not able to completely displace DGDG from the interface, which may explain why DGDG have an inhibitory effect on lipase activity even in the presence of bile salts at a physiological concentration. These findings could lead to the production of novel food emulsions designed for regulating dietary fat digestion and absorption in the prevention and treatment of obesity and related disorders. In line with these findings, Erlanson-Albertsson et al. have given orally a preparation of thylakoids from green leaves to mice<sup>208</sup> and human healthy volunteers<sup>209</sup> to slow down lipid digestion and trigger satiety mechanisms. They have found that the ingestion of thylakoids mixed with a high-fat

meal could induce the release of gut hormones like CCK and leptin. In mice, satiety was promoted and hunger suppressed, leading to a loss of body weight and body fat. Based on *in vitro* digestion experiments, Erlanson-Albertsson et al. assumed that the mechanism by which thylakoids act is a reduction of the rate of intestinal lipolysis by pancreatic lipase, which allows some lipolysis products to reach the distal intestine and trigger the release of satiety hormones<sup>210</sup>. However, the hypothesis that galactolipids, either purified or originating from thylakoids, could slow down intestinal lipolysis was based on *in vitro* digestion studies performed with classical pancreatic lipase alone<sup>206</sup>. The possible hydrolysis of galactolipids by the digestive galactolipases PLRP2 and CEH was not taken into account. Indeed, the effects of galactolipids could be changed or suppressed under conditions involving all the lipolytic enzymes naturally present in pancreatic secretion. The recent *in vitro* study on CRF digestion by human pancreatic juice shows that galactolipids from CRF can be rapidly hydrolyzed by pancreatic enzymes during the intestinal phase of digestion<sup>109</sup>. Thus, some other components from thylakoids may be responsible of the satiety effects observed by Erlanson-Albertsson et al.<sup>210</sup>.

The development of CRF as novel food or feed ingredients may facilitate the intake of galactolipid PUFA from plants that are not conventionally consumed by humans and some animal species, such as grass and green waste residues from agriculture. The Lipid Group in the School of Biosciences at the University of Nottingham, UK, has developed a process for the recovery of the chloroplast-rich fractions from green materials using mild disruption of plant cells in 0.3 M sucrose solution<sup>145</sup>, as well as a physical fractionation using a slow-screw twin gear juicer without added water or chemicals<sup>110</sup>. The nutrient-rich juice obtained is then separated from the fibrous pulp by filtration and centrifugation, before the pellet containing CRF is freeze dried for storage. Additional post-harvest steps of blanching, steam sterilisation and pasteurization can be added to avoid galactolipid hydrolysis and fatty acid oxidation by

endogeneous enzymes<sup>109</sup>. The possibility of scaling-up this process was investigated using pea vine haulm<sup>109</sup> and these studies have paved the way for a realistic production of CRF at an industrial scale.

An amazing finding about CRF production process is its analogy with juicing procedures already established in gastronomy to fully valorize green vegetables. In order to save green wastes and the nutrients they contain, chefs have elaborated recipes to cook parts of vegetables that are usually discarded<sup>211</sup>. Pea pods and their juice are for instance used in many recipes<sup>211</sup>. The preparation of green vegetable juices often involves blanching for 3-4 minutes, prior to juicing using blender or juicer, and finally filtration to remove fibers. The juices thus obtained can be cooked in various ways such as soups, mixed with cream to make scums or mixed with fruit like apple and lemon juice to make smoothies. So far, however, nutritional facts about commercially available green juices are totally ignoring the presence of galactolipids and their fatty acids and indicate fat contents equal to zero.

CRF are however enriched in proteins (18 to 44 % DW), lipids (29 to 36 % DW), micronutrients ( $\alpha$ -tocopherol,  $\beta$ -carotene, lutein), minerals (K, Ca, P, Na, Mg, Fe) and essential FAs (mainly ALA) compared to the whole plant<sup>145</sup>. For instance, CRF prepared from grass (*Paspalum notatum*) show one of the highest content in ALA (69.5 mg/g DW versus 44.4 mg/g DW in whole plant leaves)<sup>145</sup>, while CRF prepared from pea vine haulm contain  $14.29 \pm 2.06$  mg ALA per g DW<sup>109</sup>. For comparison, CRF prepared from blanched spinach leaves contain  $35.56 \pm 2.56$  mg ALA per g DW<sup>109</sup>. The partial replacement of fish diet by CRF has shown an increase of the ALA levels in the whole body of zebrafish<sup>144</sup> and thus confirmed the potential of CRF to provide ALA. Moreover, the use of CRF allows reducing the n-6 to n-3 FA ratio (Table 6), a parameter that is essential for normal growth and development. This parameter has increased in modern human diets compared to the diet of our ancestors that was rich in fruit, vegetables, lean meat, and fish<sup>212</sup>. In Western countries, it

is today recommended to decrease the intake of n-6 FAs and increase the intake of n-3 FAs to limit cardiovascular diseases and other chronic diseases <sup>213</sup>. Galactolipids from CRF that contain high levels of ALA (n-3) but also low levels of LA (n-6), appear to be an appropriate dietary supplement to reach this recommendation, apart from restoring nutritional habits close to those of our ancestors. The proportion of galactolipids in the diet of hunter-gatherers was certainly higher than it is in modern diets because they were eating more fruit and vegetables. Moreover, they were eating wild herbivores that had certainly higher levels of ALA in their meat.

Today, ALA supplementation is mostly ensured by the consumption of ALA-rich vegetable oils, such as flaxseed oil <sup>214, 215</sup>. Recent studies have shown however that the high susceptibility to oxidation of ALA-enriched emulsions could be harmful for human health and therefore, their incorporation into functional food is still limited <sup>216</sup>. Stability of ALA in CRF has not been extensively studied so far, but the high contents of antioxidant molecules ( $\beta$ -carotene,  $\alpha$ -tocopherol, lutein) in chloroplast may be beneficial for preserving ALA from oxidation.

Another interesting finding from the CRF supplementation trial made in zebrafish is that the levels of EPA and DHA in the fish body are preserved when 50 % w/w of the fish diet containing EPA and DHA is replaced by CRF (Table 6) <sup>144</sup>. This suggests that part of the ALA from CRF, and therefore from galactolipids, is efficiently converted into longer chain PUFA <sup>217</sup>.

## **11. Conclusions**

We hope that this review will trigger the interest for the digestion of galactolipids in various species. First, because galactolipids represent the main source of the essential fatty acid ALA,

and their digestion for the release and intake of ALA appears as a ubiquitous process in the living world. Second, many herbivorous species utilise galactolipids, not only for the intake of essential PUFA but also as the main source of fatty acids and energy for growth.

Many herbivorous species have solved the challenge of concentrating the galactolipid fatty acids dispersed in the green biomass by eating continuously grass or leaves. Since these plant resources contain a lower density of energy than other types of foods, herbivores tend to have longer digestive tracts with slow metabolisms to optimise the digestion processes. It was shown for instance that zebrafish fed with a herbivore diet have the longest guts, the largest intestinal epithelial surface area and enterocyte cellular volumes compared to zebrafish fed with a carnivore or omnivore diet <sup>148</sup>. Horses have an entire digestive tract that measures nearly 30 meters including a 20-meters long small intestine that precedes the cecum and large intestine where fermentation of plant fibers occurs. Horses are “hindgut” fermenters and differ from “foregut” fermenters such as ruminants (cattle, sheep, goats), in which fermentation mainly occurs in the upper GI tract (rumen). The horse GI tract anatomy thus allows the release and intake of galactolipid PUFA before they could be degraded by the microbiota in the lower GI tract or hindgut.

As observed in insects, another adaptation to the herbivore diet is the production of many digestive enzymes that ensures a high and efficient breakdown of the diet. This was known for the digestion of dietary fibers and carbohydrates from plants, but the huge number of lipolytic enzymes (PLRPs) produced in the midgut of foliovorous insects, as well as the large increase in their expression observed when these insects are eating leaves, support a similar adaptation for the digestion of plant lipids <sup>162, 163</sup>. Similarly, a herbivorous fish like the monkey face prickleback (*Cebidichthys violaceus*), has several genes coding for CEH-like enzymes, resulting from gene duplication events and these genes are highly expressed in the middle intestine <sup>154</sup>. It is likely that these species invest in the production of galactolipases to

ensure lipid digestion from their plant leaves or algal diet in which galactolipids are dispersed and the density of FAs is limited compared to the TAG fatty acids stored in intracellular lipid droplets. These adaptations are in line with the nutrient balancing hypothesis, according to which animals invest in the synthesis of digestive enzymes to acquire limiting nutrients <sup>155</sup>.

In terms of potential applications in nutrition, a better understanding of galactolipid digestion is highly relevant for the breeding of herbivorous animal species and aquaculture. The digestion of galactolipids could also be an interesting target to fight against agriculture pests such as foliovorous moths and phytopathogenic fungi, which need ALA for their development. With respect to humans, the fact that we can digest galactolipids brings us back to our origins. It reminds us that we should eat vegetables to have access to the essential ALA and restore the right balance of anti-inflammatory (n-3) versus pro-inflammatory (n-6) fatty acids. Our modern diet contains too much n-6 fatty acids due to the industrial processing of vegetable oils and the selective loss of ALA on one hand, and due to the increased use of intensive, cereal-based livestock production systems that have resulted in a lower proportion of n-3 fatty acids in meat compared with traditional extensive production systems, on the other hand <sup>218</sup>. Apart from eating more vegetables, changing breeding systems and adapting oils and fats industrial processes, novel dietary supplements like CRF <sup>145, 219</sup> may help us to re-equilibrate the n-3/n-6 balance. Additionally, unconventional plant resources could be used for human nutrition <sup>145</sup>.

The digestion of galactolipids may also offer the possibility to study the evolution of dietary habits and the adaptation of species to herbivorous diet and novel environments <sup>149, 154</sup>.

Paleodietary studies based on stable carbon isotope analysis and <sup>13</sup>C/<sup>12</sup>C ratio in tooth enamel of extinct animals have revealed the photosynthetic pathway from which dietary carbon was derived over various period of time. They have shown that four million years ago, early hominins from southern Africa had diets that were dominated by plant resources using C3



photosynthesis to harvest CO<sub>2</sub> and initiate biomolecules synthesis via the Calvin Benson Bassham cycle in the chloroplast. C3 plants (including fruit, leaves, and the roots of trees, bushes, shrubs and forbs) have a lower propensity to discriminate against <sup>13</sup>C isotope during fixation of CO<sub>2</sub> than do C4 plants such as tropical grasses and sedges (including blades, seeds, and roots)<sup>220</sup>. Hominins had therefore a diet similar to that of extant chimpanzees eating fruit and leaves. By about 3.5 Ma, there is a trend toward greater consumption of <sup>13</sup>C-enriched foods in early hominins over time, which suggests that either they have expanded their dietary habits by eating grass or they began eating other animals that ate grass<sup>221</sup>. It is likely that the <sup>13</sup>C signature of human dietary habits and evolution has a direct relationship with the ability to digest galactolipids, the main lipids present in plants and therefore in the diet of early hominins.

## **Acknowledgements**

We are grateful to Regine Lebrun, Pascal Mansuelle and Kaouthar Dridi for the mass spectrometry analysis of the proteins from the midgut of *Helicoverpa armigera* at the proteomics facilities of the Mediterranean Institute of Microbiology (FR 3479 IMM, CNRS, Marseille Protéomique), Marseille, France.

## **Conflict of interest statement**

Sawsan Amara, PhD, is the founder and CEO of Lipolytech, a company producing digestive enzymes. All other authors do not have conflict of interest to declare.

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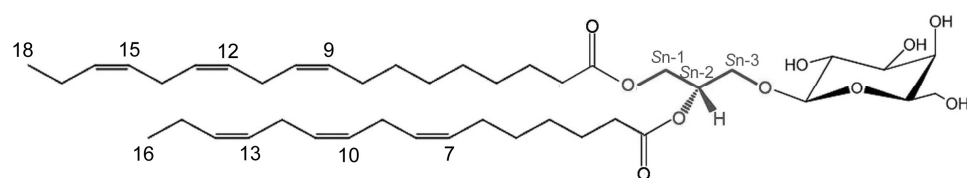


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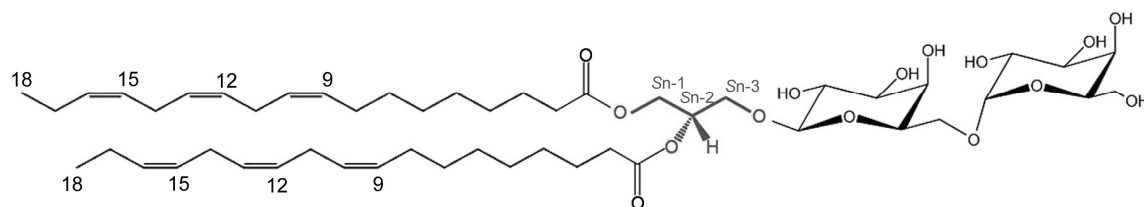
a newly isolated *Fusarium solani* strain, *Appl Biochem Biotechnol*, 2012, **168**, 2330-2343.

## Figures

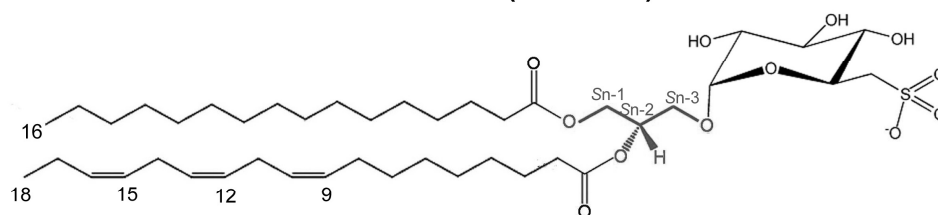
Figure 1. Chemical structures of 1,2-diacyl-3-O- $\beta$ -D-galactosyl-*sn*-glycerol (MGDG), 1,2-diacyl-3-O-(6-O- $\alpha$ -D-galactosyl- $\beta$ -D-galactosyl)-*sn*-glycerol (DGDG), and of 1,2-diacyl-3-(6-sulfo- $\alpha$ -D-quinovosyl)-*sn*-glycerol (SQDG). We represented here a MGDG molecule with 18:3/16:3 fatty acids at sn-1 and sn-2 positions, respectively, and a DGDG molecule with two 18:3 fatty acids at sn-1 and sn-2 positions which are predominant molecular species of prokaryotic and eukaryotic galactolipids, respectively, in spinach leaves<sup>47</sup>, as well as a SQDG molecule with 16:0 and 18:3 fatty acids at sn-1 and sn-2 positions, the most represented molecular species of SQDG in spinach leaves<sup>222</sup>.



**MGDG (18:3/16:3)**



**DGDG (18:3/18:3)**



**SQDG (16:0/18:3)**

Figure 2. Schematic representation of plant cells from leaves and their chloroplasts. N, nucleus; V, vacuole. Chloroplasts contain several membranes, including the outer and inner envelopes and the thylakoid membranes where photosynthesis occurs. Galactolipids are the main constituents of these membranes.

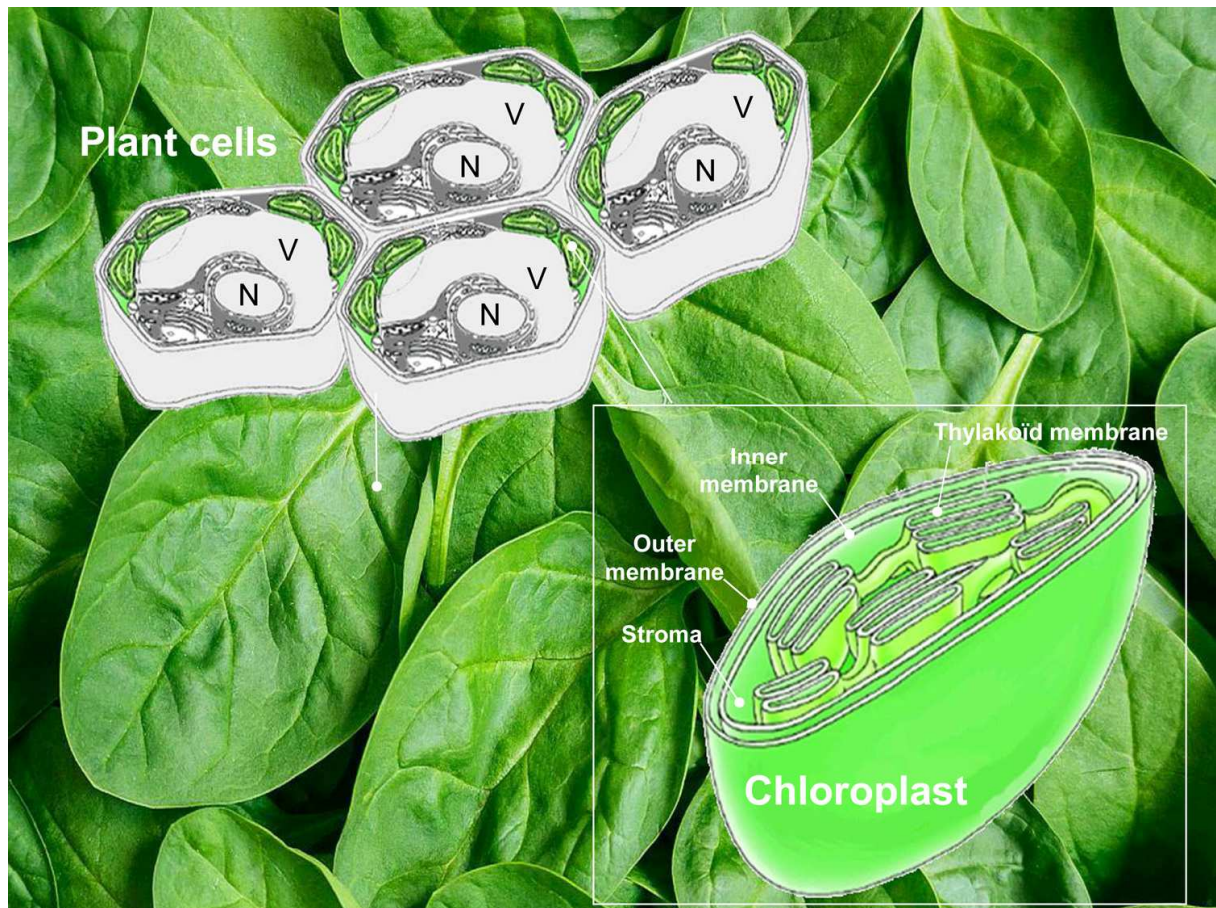


Figure 3. Schematic representation of galactolipid synthesis and transport in the chloroplast. The prokaryotic (chloroplast) and eukaryotic (ER) pathways of diacylglycerol (DAG) synthesis are shown with their distinct specificity for the incorporation of acyl chains at the *sn*-2 and *sn*-1 positions of glycerol. Abbreviations: ACP, acyl carrier protein ; CoA, coenzyme A; AAPT, cytidine-diphosphate (CDP)-choline:DAG phosphocholine transferase; DGAT, diacylglycerol acyltransferase; DGD, DGDG synthase; ER, endoplasmic reticulum; GAP, Glycerol-3-phosphate acyltransferase; KAS, 3-ketoacyl-ACP synthase; LPA, lysophosphatidic acid; LPAAT, LPA acyltransferase; MGD, MGDG synthase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol-phosphate synthase; PGPP, phosphatidylglycerol-phosphate phosphatase; SAD, stearyl-acyl carrier protein  $\Delta^9$  desaturase; SQD1, UDP-sulfoquinovose synthase; SQD2, UDP-sulfoquinovose:DAG Sulfoquinovosyltransferase; TAG, triacylglycerol. The respective levels of MGDG, DGDG, SQDG, PG and PC in thylakoïd, Inner and Outer membranes are expressed in % of total lipids. Adapted from <sup>68</sup>.

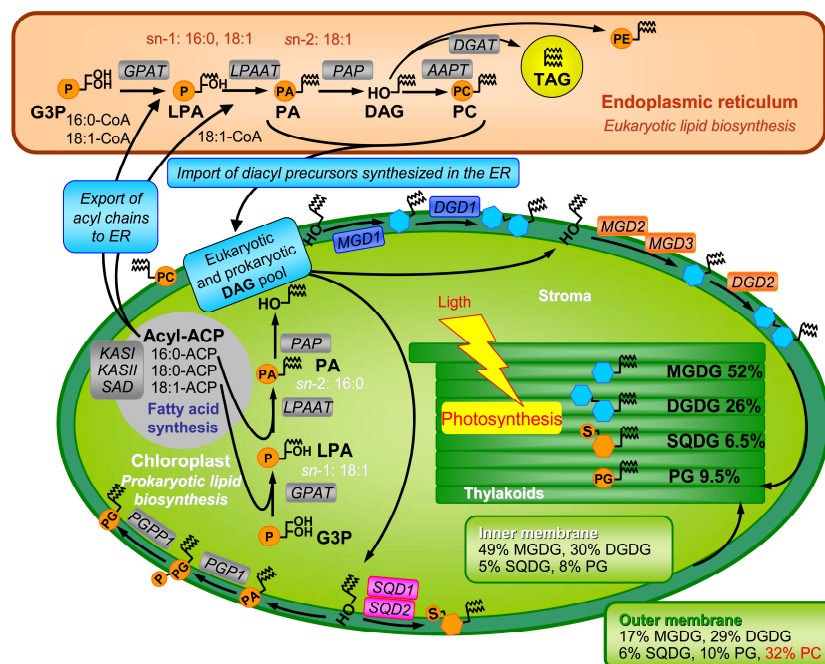


Figure 4. *Galactolipid synthesis and fatty acid desaturation in plants*. Some desaturation of oleic acid (18:1) into linoleic acid (18:2) and linolenic acid (18:3) already occurs in the ER through the action of FAD2 and FAD3 fatty acid desaturases. In the chloroplast, MGDG are produced by MGDG synthases (MGD) via transfer of a galactose from UDP-galactose onto DAG molecules originating from the prokaryotic/chloroplastic (DAG-18:1,16:0) or eukaryotic/ER (DAG-18:2,18:2, DAG-16:0,18:2) pathways. Then, DGDG synthase (DGD) convert MGDG into DGDG by transfer of a second galactose from UDP-galactose. Several chloroplastic desaturases (FAD5, FAD6, FAD7, and FAD8) introduce double bonds in MGDG and DGDG. Adapted from <sup>68</sup>.

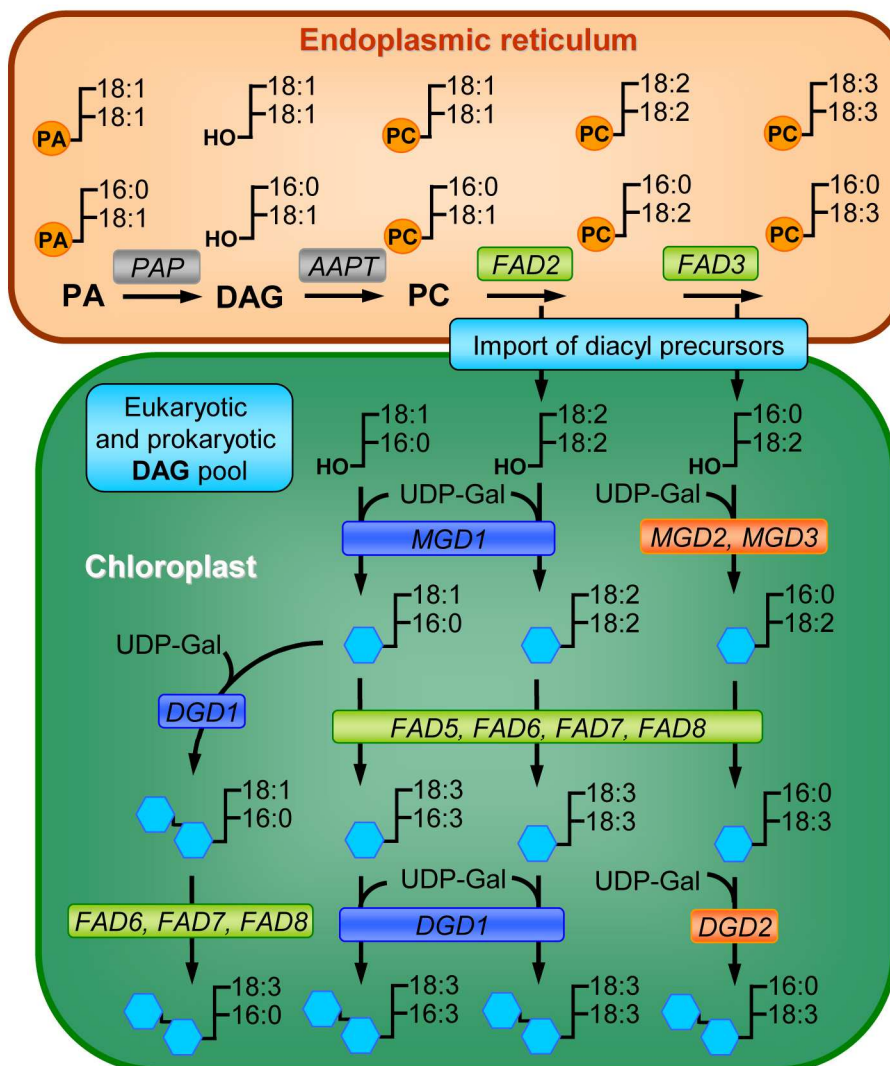


Figure 5: Enzymatic hydrolysis of a DGDG molecule by galactolipases, with the release of one digalactosylmonoacylglycerol (DGMG) and one FFA molecules. Most galactolipases characterized so far have a *sn*-1 regioselectivity with a preferential hydrolysis of the ester bond found at the *sn*-1 position of the glycerol backbone<sup>63, 67</sup>.

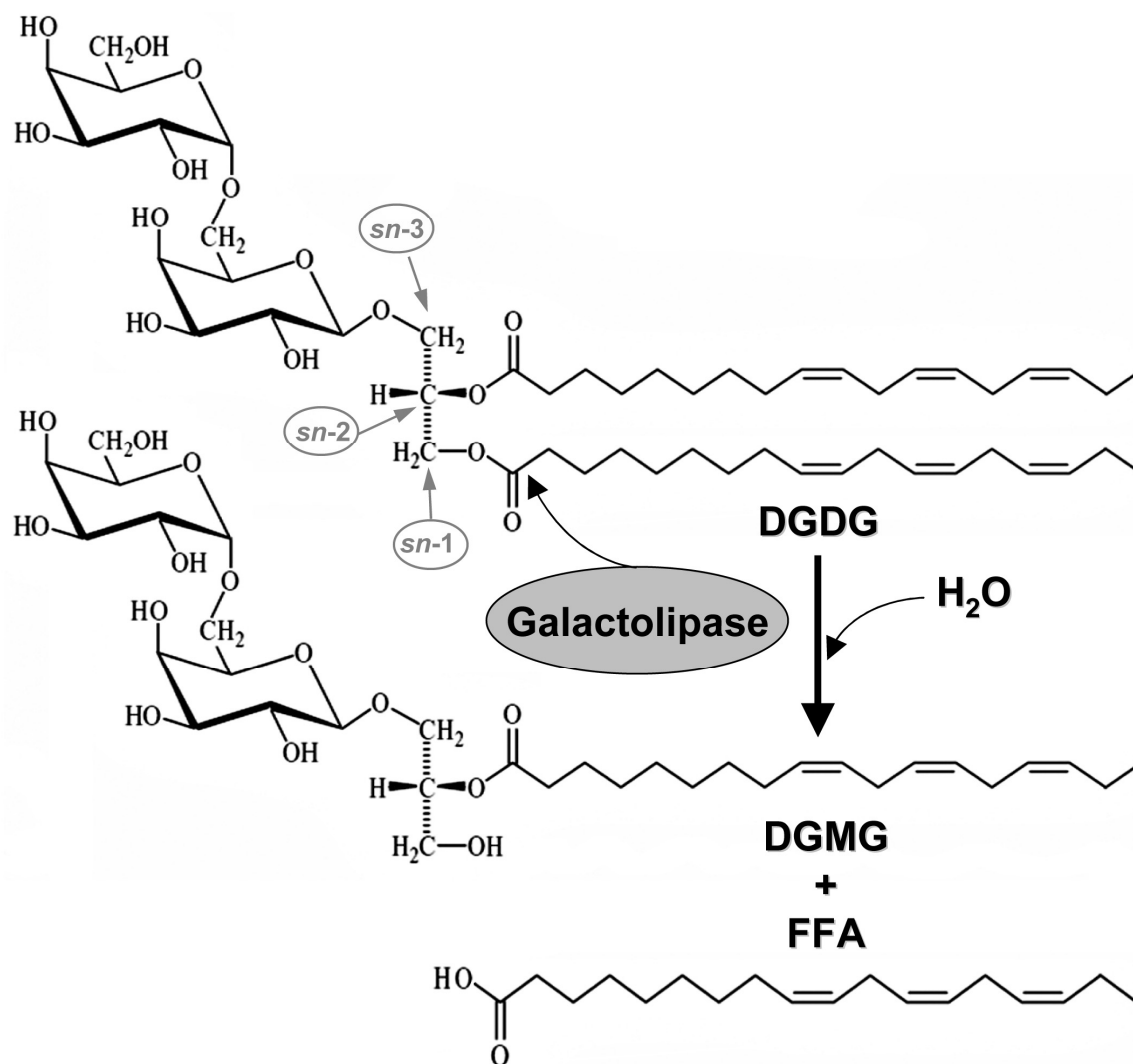


Figure 6: Protein sequence alignment of human PLRP2 (HPLRP2; UniProtKB/Swiss-Prot: P54317), guinea pig PLRP2 (GPLRP2; NCBI Accession ID: NP\_001177220) and a pancreatic lipase-related protein (HaPLRP) identified in the midgut of *Helicoverpa armigera* larvae (NCBI Reference Sequence: XP\_021199604.1, derived from the genomic sequence of *Helicoverpa armigera* NW\_018396066.1<sup>163</sup>). Sequence alignment was performed using the Clustal Omega web server (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)<sup>223</sup>. Secondary structure elements shown above the sequence alignment were obtained from the known 3D structure of human PLRP2 (PDB: 2PVS;<sup>84</sup>) and the DSSP software<sup>224</sup> accessible via the web server: <http://www.cmbi.ru.nl/xssp/>. The presentations of sequence alignments were made using ESPrnt 3.0 (<http://esprnt.ibcp.fr/>)<sup>225</sup>. The peptides of HaPLRP identified by trypsin digestion and mass spectrometry are underlined by a grey line. The residues of the catalytic triad (Ser152-Asp176-His-263 in HPLRP2) are indicated by stars.

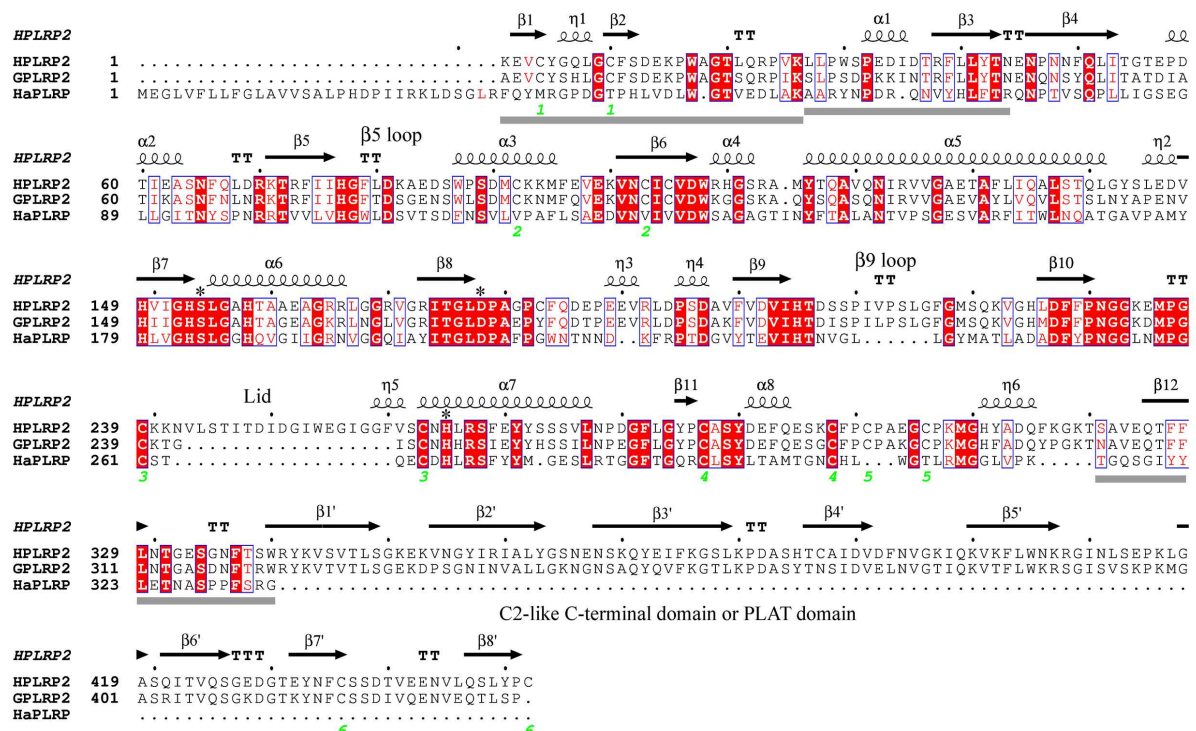




Figure 7: Molecular docking of a 18:3 *n*-3/18:3 *n*-3 DGDG molecule in the active site of pancreatic lipase-related proteins and *Fusarium solani* cutinase. Panel A: X-ray crystallography 3D structure of HPLRP2 (PDB ID: 2PVS<sup>84</sup>); Panel B: X-ray crystallography 3D structure of a chimera made of the N-terminal catalytic domain of GPLRP2 and the C-terminal domain of human pancreatic lipase (PDB ID: 1GPL<sup>83</sup>); Panel C: X-ray crystallography 3D structure of *Fusarium solani* cutinase (PDB ID: 1CEX<sup>226</sup>); Panel D: 3D homology model of *Helicoverpa armigera* pancreatic lipase-related protein (HaPLRP). The 3D model of HaPLRP was generated using the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html>)<sup>227</sup> and was built from the known 3D structure of HPLRP2 (2PVS) and the sequence alignment shown in Figure 6. The Autodock vina program<sup>228</sup> was used for molecular docking of DGDG and was executed in the structure viewing software PyMol (PyMOL Molecular Graphics System, Version 1.3. Schrödinger, LLC; <http://www.pymol.org/>) via a plugin for in silico docking<sup>229</sup>. Pictures of the 3D models were generated using PyMOL and are presented as molecular surfaces with the following color codes (white: C and H atoms; blue: N atoms; red: O atoms; yellow: S atoms). The DGDG is presented as sticks model. The locations of the two acyl chains (*sn*-1 and *sn*-2) and the digalactosyl polar head (*sn*-3) on the glycerol backbone are indicated according to the stereospecific numbering nomenclature.

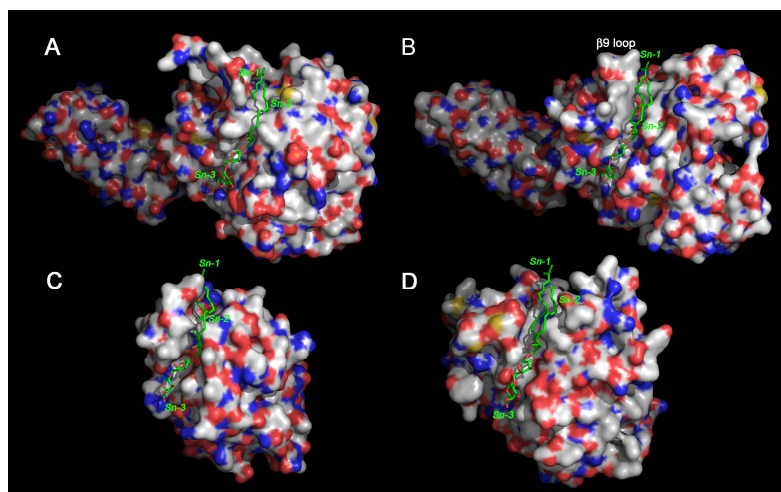


Figure 8: Superimposition of the 3D structures of truncated human pancreatic carboxyl ester hydrolase (HCEH; PDB: 1JMY; <sup>147</sup>) and human PLRP2 (PDB:PVS <sup>84</sup>), based on their common  $\alpha/\beta$  hydrolase fold. The truncated HCEH (Ala1-Phe518) does not contain the C-terminal tandem repeat region found in the full length mature protein (722 amino acid residues). The picture was generated using PyMOL and the molecular structures are represented by their C $\alpha$  tracing and secondary structure elements. The active site serine found in the nucleophilic elbow of the  $\alpha/\beta$  hydrolase fold is indicated in red.

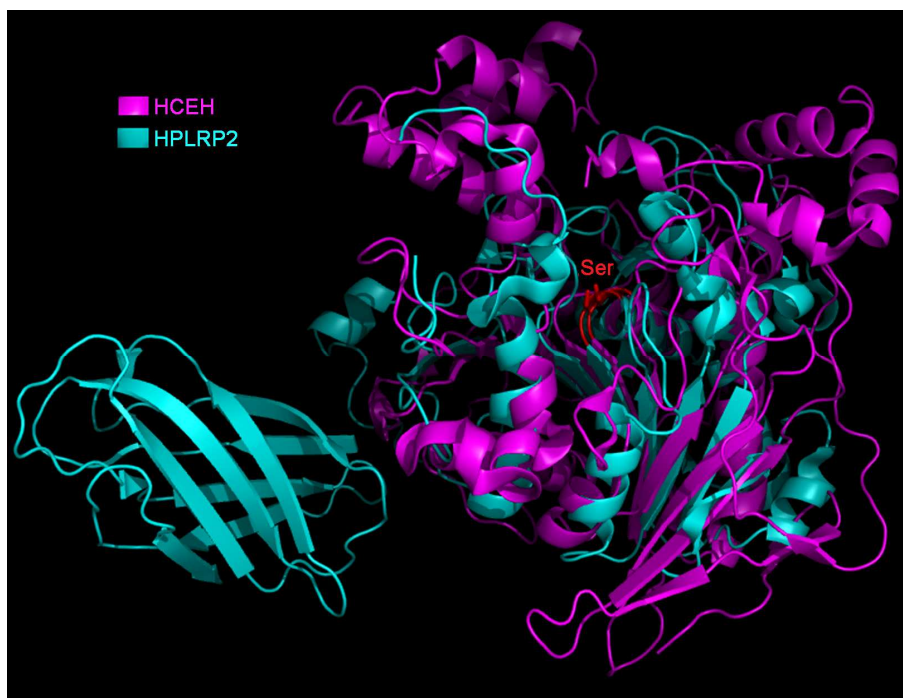


Figure 9: Close-up views of Molecular docking of a 18:3 n-3/18:3 n-3 DGDG molecule in the active site of pancreatic carboxyl ester hydrolases from various species. Panel A: DGDG docking in the X-ray crystallography 3D structure of truncated human CEH (PDB ID: 1JMY<sup>147</sup>); Panel B: DGDG docking in X-ray crystallography 3D structure of bovine CEH (PDB ID: 1AQL ;<sup>230</sup>), co-crystallized in the presence of sodium taurocholate (TC; shown as orange stick model); Panels C and D: 3D homology models of zebrafish (*Danio rerio*) carboxyl ester hydrolase tandem duplicates 1 and 2, generated using the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html>)<sup>227</sup> and their respective sequences (Genebank AAH55668 and NCBI Reference Sequence NP\_001020344.2) shown in Figure 10. The Autodock vina program<sup>228</sup> was used for molecular docking of DGDG and was executed in the structure viewing software PyMol (PyMOL Molecular Graphics System, Version 1.3. Schrödinger, LLC; <http://www.pymol.org/>) via a plugin for in silico docking<sup>229</sup>. Pictures of the 3D models were generated using PyMOL and are presented as molecular surfaces with the following color codes (white: C and H atoms; blue: N atoms; red: O atoms; yellow: S atoms). The DGDG is presented as green sticks model. The locations of the two acyl chains (*sn*-1 and *sn*-2) and the digalactosyl polar head (*sn*-3) on the glycerol backbone are indicated according to the stereospecific numbering nomenclature.

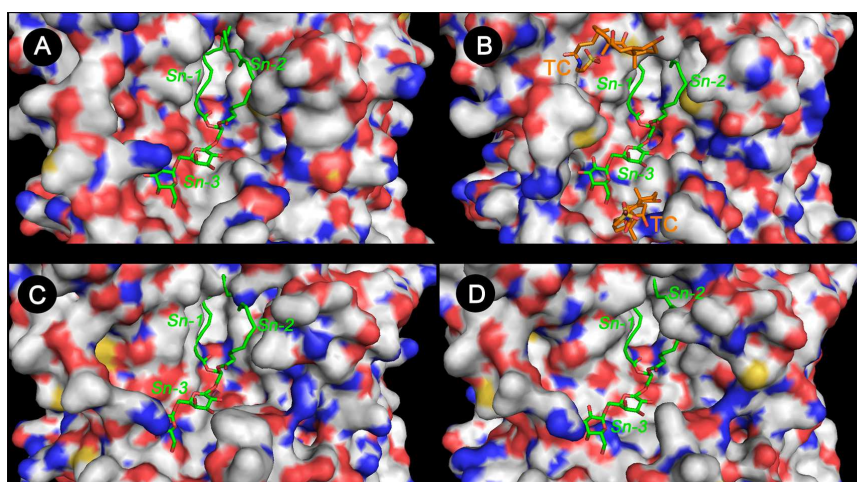


Figure 10: Protein sequence alignment of human pancreatic carboxyl ester hydrolase (HCEH/BSSL; UniProtKB/Swiss-Prot: P19835.3) and the two tandem duplicates of zebrafish (*Danio rerio*) carboxyl ester hydrolases (DrCEH1; Genebank AAH55668 for tandem duplicate 1) and DrCEH2 (NCBI Reference Sequence NP\_001020344.2 for tandem duplicate 2). Sequence alignment and presentation were performed as indicated in Figure 6 legend. Secondary structure elements shown above the sequence alignment were obtained from the known 3D structure of truncated human CEH/BSSL (PDB: 1JMY; <sup>147</sup>). The residues of the catalytic triad (Ser194-Asp 320-His-435 in HCEH) are indicated by stars and the vertical arrows indicate the cleavage site of the signal peptide.

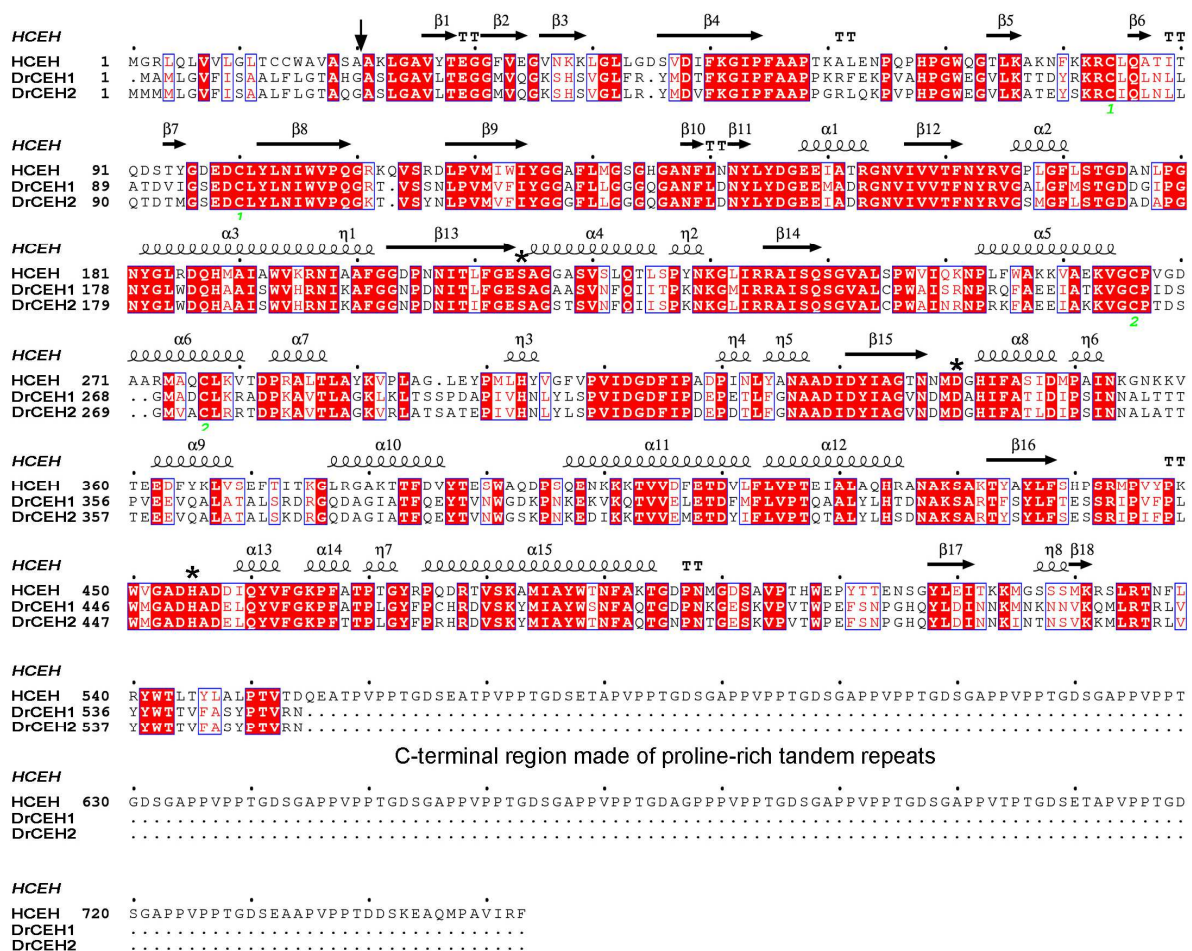


Figure 11: *Hydrolysis of galactolipids in the course of two-step in vitro digestion of CRFs prepared from blanched spinach leaves.* CRFs were first mixed with rabbit gastric extracts (RGE) and incubated at pH 5 and 37°C for 30 minutes (gastric phase) and then mixed with human pancreatic juice (HPJ) and bile salts (4 mM NaTDC), and further incubated at pH 6 for 60 minutes (intestinal phase). Panel A: time-course TLC analysis of polar lipids in which galactolipids are revealed with thymol. Pure MGDG and DGDG are used as reference standards. The lipolysis products, MGMG and DGMG, appearing upon hydrolysis are indicated by white arrows. Panel B: time-course quantitation of residual MGDG and DGDG, and MGMG appearing during the two-step *in vitro* digestion. The respective amounts of galactolipids were estimated from scanning densitometry of the TLC plate in panel A and calibration curves established with reference standards. Panel C: time-course TLC analysis of neutral lipids stained with copper acetate-phosphoric acid. Oleic acid was used as reference standard for free fatty acids (FFA). Panel D: time-course quantitation of FFA appearing during the two-step *in vitro* digestion. The amounts of FFA were estimated from scanning densitometry of the TLC plate in panel B and a calibration curve established with oleic acid. Values (mg of galactolipid or FFA per g of CRF dry weight) are means  $\pm$  SD (n=3).

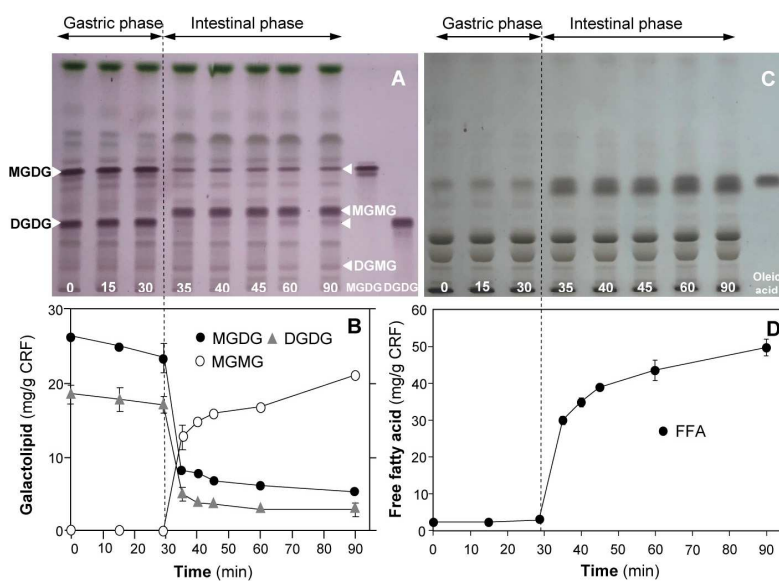


Figure 12: Variations with diet in the galactolipase activity (panel A; U per mg of fresh weight) and galactolipase to lipase activity ratio (panel B) in the midgut of the Lightbrown Apple Moth *Epiphyas postvittana*, the Cotton Bollworm *Helicoverpa armigera*, the Indian Meal Moth *Plodia interpunctella*, the Mediterranean Flour Moth *Ephestia kuehniella*, the Greater Wax Moth *Galleria mellonella* and the Common Clothes Moth *Tineola bisselliella*. The galactolipase and lipase activities were measured using the pH-stat technique, the medium-chain C8-MGDG and trioctanoin as substrate, respectively. Adapted from <sup>124</sup>.

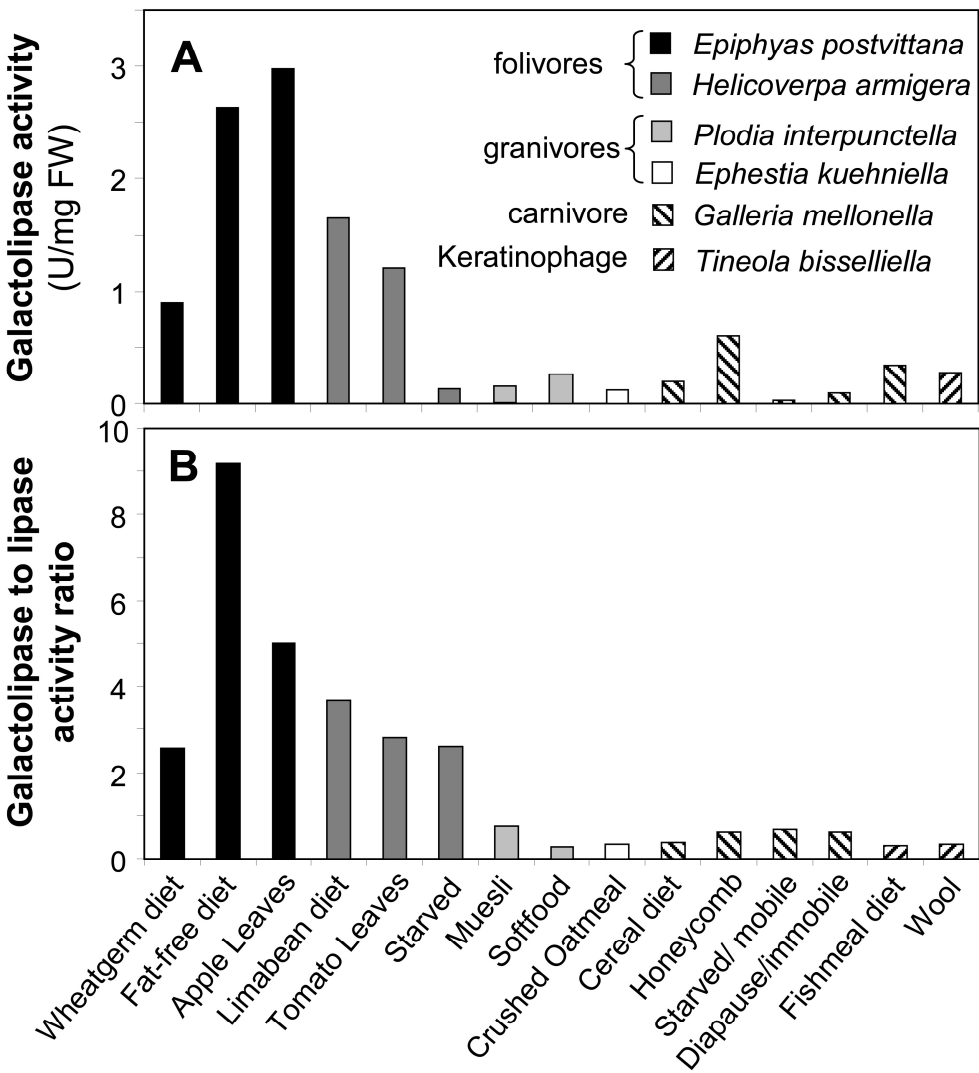


Figure 13: effects of N-linolenyl-L-glutamate (NLLG) on the phospholipase (panel A) and galactolipase (B) activities from *Helicoverpa armigera* larval midgut. Enzyme assays were performed using egg phosphatidylcholine and C8-MGDG as substrates according to <sup>124</sup>, except that bile salts were replaced by NLLG at various concentrations.

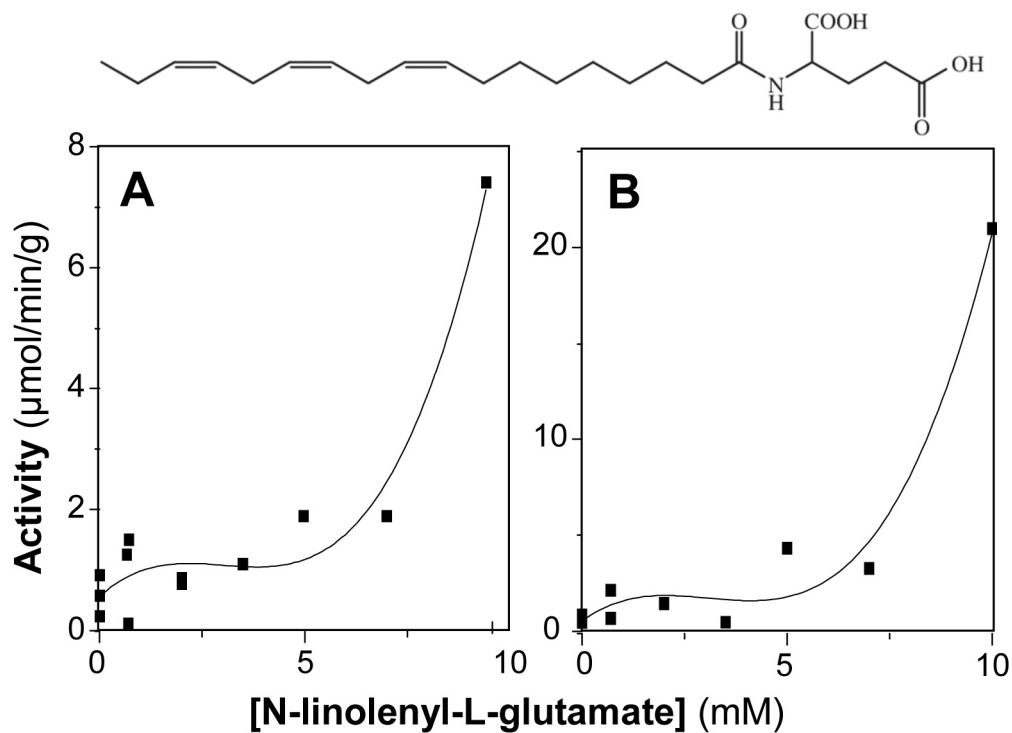


Figure 14: Protein sequence alignment of fungal lipases with galactolipase activity.

Alignment was performed using the Clustal Omega web server (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)<sup>223</sup> using the sequences of *Gibberella zeae* (*Fusarium graminearum*) lipase (GZEL/FGL1; GenBank: AAQ23181.1), *Fusarium solani* (*Nectria haematococca*) lipase (FSL; NCBI Reference Sequence: XP\_003050606.1), *Thermomyces lanuginosus* lipase (TLL; UniProtKB/Swiss-Prot: O59952.1) and *Talaromyces thermophilus* lipase (TTL; GenBank: AEE61324.1). Secondary structure elements shown above the sequence alignment were obtained from the known 3D structure of GZEL (PDB: 3NMG;<sup>205</sup>) and the DSSP software<sup>224</sup> accessible via the web server: <http://www.cmbi.ru.nl/xssp/>. The presentations of sequence alignments were made using ESPript 3.0 (<http://esprict.ibcp.fr/>)<sup>225</sup>. The residues of the catalytic triad (Ser-Asp-His) are indicated by stars.

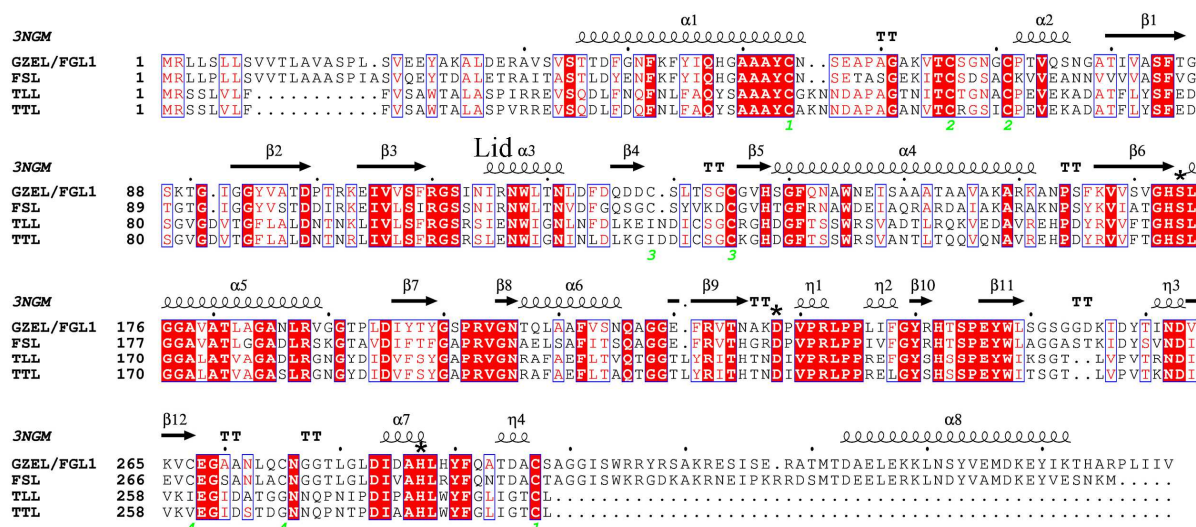
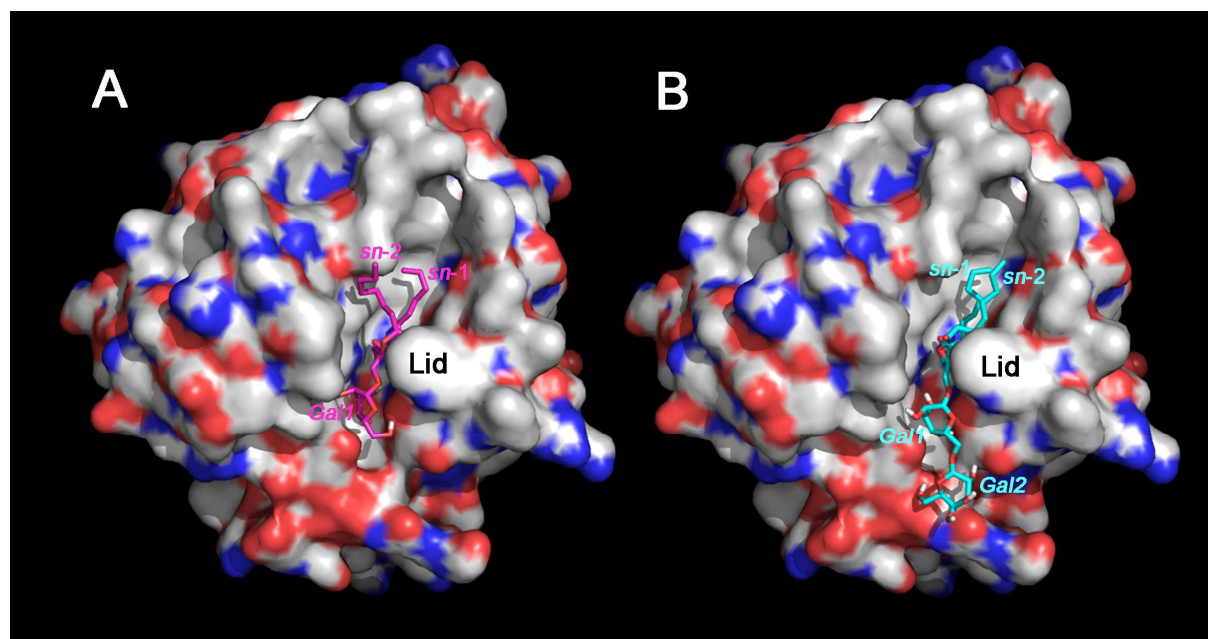




Figure 15: Molecular docking of C8-MGDG (panel A) and C8-DGDG (panel B) molecules in the active site of *Fusarium solani* lipase (FSL). The 3D homology model of FSL was generated using the known structures of GZEL (PDB: 3NMG<sup>205</sup>) and TLL (PDB: 1GT6<sup>204</sup>) and sequence alignment in Figure 14. The Autodock vina program<sup>228</sup> was used for molecular docking of MGDG and DGDG and was executed in the structure viewing software PyMol (PyMOL Molecular Graphics System, Version 1.3. Schrödinger, LLC; <http://www.pymol.org/>) via a plugin for in silico docking<sup>229</sup>. Pictures of the 3D models were generated using PyMOL and are presented as molecular surfaces with the following color codes (white: C and H atoms; blue: N atoms; red: O atoms; yellow: S atoms). The C8-MGDG (panel A) and C8-DGDG (panel B) are presented as pink and cyan sticks models, respectively. The locations of the two acyl chains (*sn*-1 and *sn*-2) and the galactosyl groups (gal1 and gal 2) at the *sn*-3 position of the glycerol backbone are indicated.



**Table 1:** MGDG and DGDG contents in some vegetables and fruit. Values are mg/ Kg of fresh weight (FW)\* or dry weight (DW)\*\*. CRF, Chloroplast-rich fraction. Adapted from <sup>231</sup>.  
<sup>a</sup>Data from <sup>232</sup>; <sup>b</sup>Data from <sup>233</sup>; <sup>c</sup>Data from <sup>234</sup>; <sup>d</sup>Data from <sup>109</sup>; <sup>e</sup> Estimated from values in <sup>112</sup> for total galactolipids.

| Vegetables leaves  |                                       | MGDG  | DGDG                        |
|--------------------|---------------------------------------|---|-----------------------------|
| Parsley            | <i>Petroselinum crispum</i>           | 1838* <sup>a</sup>  | 1157* <sup>a</sup>          |
| Leek               | <i>Allium ampeloprasum var. porum</i> | 778* <sup>a</sup>   | 588* <sup>a</sup>           |
| Spinach            | <i>Spinacia oleracea</i>              | 546* <sup>b</sup> ; 850* <sup>a</sup> ; 3300-38800** <sup>c</sup> | 563* <sup>a</sup>           |
| Spinach CRF        | <i>Spinacia oleracea</i>              | 28590 ± 1120** <sup>d</sup>                                       | 25810 ± 1120** <sup>d</sup> |
| Lettuce            | <i>Lactuca sativa</i>                 | 135* <sup>a</sup> ; 32-320* <sup>b</sup>                          | 148* <sup>a</sup>           |
| Cabbage            | <i>Brassica oleracea</i>              | 105* <sup>a</sup>   | 83* <sup>a</sup>            |
| Red clover         | <i>Trifolium pratense</i>             | 1795* - 14480** <sup>e</sup>                                      |                             |
| Legumes            |                                       |   |                             |
| Pea                | <i>Pisum sativum var. sativum</i>     | 22** <sup>a</sup> ; 239-442* <sup>b</sup>                         | 698** <sup>a</sup>          |
| Pea vine haulm CRF | <i>Pisum sativum var. sativum</i>     | 18890 ± 1970** <sup>d</sup>                                       | 13220 ± 990** <sup>d</sup>  |
| kydney bean        | <i>Phaseolus vulgaris</i>             | 396* <sup>b</sup> ; 230* <sup>a</sup>                             | 211* <sup>a</sup>           |
| Stem vegetables    |                                       |   |                             |
| Asparagus          | <i>Asparagus officinalis</i>          | 262* <sup>a</sup>   | 225* <sup>a</sup>           |
| Broccoli           | <i>Brassica oleracea var. italica</i> | 316* <sup>b</sup> ; 377* <sup>a</sup>                             | 242* <sup>a</sup>           |
| Root vegetables    |                                       |   |                             |
| Carrot             | <i>Daucus carota ssp. sativus</i>     | 40* <sup>a</sup>  | 66* <sup>a</sup>            |
| Potato             | <i>Solanum tuberosum</i>              | 19* <sup>a</sup> ; 41* <sup>b</sup>                               | 47* <sup>a</sup>            |
| Sweet potato       | <i>Ipomoea batatas</i>                | 97* <sup>a</sup>  | 226* <sup>a</sup>           |
| Fruit vegetables   |                                       |   |                             |
| Pumpkin            | <i>Cucurbita maxima</i>               | 617*  | 686*                        |
| Cucumber           | <i>Cucumis sativus</i>                | 94* <sup>b</sup> ; 138* <sup>a</sup>                              | 117* <sup>a</sup>           |

**Table 2:** Galactolipid and fatty acid contents in some cereals and flour. Values are mean  $\pm$  SD (n=3). DGMG, digalactosylmonoacylglycerol; DGDG, digalactosyldiacylglycerol; MGMG, monogalactosylmonoacylglycerol; MGDG, monogalactosyldiacylglycerol. <sup>a</sup>Data from <sup>25</sup>; <sup>b</sup>Data from <sup>28</sup>.

| Sources |                                 | Galactolipids (mg/100 g) |                |               |               | Fatty acids (% w/w) |                 |                 |                 |                 |                 |                 |
|---------|---------------------------------|--------------------------|----------------|---------------|---------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|         |                                 | MGDG                     | DGDG           | MGMG          | DGMG          | 16:0                | 16:1            | 18:0            | 18:1            | 18:2            | 18:3            | others          |
| Wheat   | Spring wheat flour <sup>a</sup> | 35 $\pm$ 9.22            | 100 $\pm$ 21.0 | 24 $\pm$ 10.4 | 15 $\pm$ 2.49 | 19.5 $\pm$ 0.49     | 0.02 $\pm$ 0.01 | 0.65 $\pm$ 0.05 | 14.1 $\pm$ 0.60 | 62.6 $\pm$ 0.43 | 2.89 $\pm$ 0.73 | 0.32 $\pm$ 0.18 |
| Wheat   | Winter wheat flour <sup>a</sup> | 25 $\pm$ 3.25            | 109 $\pm$ 10.6 | 18 $\pm$ 6.34 | 23 $\pm$ 3.01 | 19.7 $\pm$ 0.51     | 0.02 $\pm$ 0.02 | 0.53 $\pm$ 0.08 | 11.5 $\pm$ 0.26 | 63.5 $\pm$ 0.37 | 4.41 $\pm$ 0.44 | 0.42 $\pm$ 0.17 |
| Oat     | Oat flour <sup>b</sup>          | 13.9 $\pm$ 7.1           | 418 $\pm$ 61   |               |               |                     |                 |                 |                 |                 |                 |                 |

**Table 3 :** Fatty acid composition (% w/w of total FA) of whole spinach leaves and galactolipids (MGDG, DGDG) purified from these leaves and relative distributions(%) of these FA at sn-1 and sn-2 positions of MGDG and DGDG. NI, non identified: nd, not detected. Adapted from<sup>67</sup>.

| <b>Fatty acids</b> | <b>Total spinach</b> | <b>MGDG</b> | <b>% of sn-1 position of MGDG</b> | <b>% of sn-2 position of MGDG</b> | <b>DGDG</b> | <b>% of sn-1 position of DGDG</b> | <b>% of sn-2 position of DGDG</b> |
|--------------------|----------------------|-------------|-----------------------------------|-----------------------------------|-------------|-----------------------------------|-----------------------------------|
| 14:0               | 0.6                  | 1.8 ± 1.1   | 50.0                              | 50.0                              | 2.7 ± 0.8   | 18.2                              | 81.8                              |
| 16:0               | 12.0                 | 6.6 ± 2.7   | 54.2                              | 45.8                              | 23.8 ± 1.4  | 36.2                              | 63.8                              |
| 16:1               | 7.6                  | 2.1 ± 0.8   | 52.6                              | 47.4                              | 4.1 ± 1.4   | 7.5                               | 92.5                              |
| 16:2               | 0.1                  | 0.1 ± 0.0   |                                   |                                   | nd          |                                   |                                   |
| 16:3               | 4.9                  | 17.3 ± 0.5  | 23.0                              | 77.0                              | 1.9 ± 0.2   | 58.7                              | 41.3                              |
| 18:0               | 1.4                  | 1.7 ± 1.1   | 62.3                              | 37.7                              | 4.1 ± 0.2   | 35.2                              | 64.8                              |
| 18:1               | 6.9                  | 3.8 ± 2.2   | 63.3                              | 36.7                              | 6.4 ± 0.1   | 45.1                              | 54.9                              |
| 18:2 n-6           | 16.1                 | 2.7 ± 0.3   | 63.3                              | 36.7                              | 6.4 ± 1     | 52.5                              | 47.5                              |
| 18:3 n-3           | 37.2                 | 60.6 ± 9.7  | 54.3                              | 45.7                              | 53.1 ± 2.6  | 65.1                              | 34.9                              |
| NI                 | 10.6                 | 3.4 ± 2.1   |                                   |                                   | 1.4 ± 2.0   |                                   |                                   |

**Table 4 :** *Galactolipase and lipase activities (U/mg) of various pancreatic lipolytic enzymes and some microbial lipases.* Galactolipase activities were measured at pH 8.0 and 37°C using the pH-stat technique and various purified galactolipids (monogalactosyl-dioctanoyl-glycerol (C8-MGDG), digalactosyl-dioctanoyl-glycerol (C8-DGDG) or natural long chain MGDG and DGDG from spinach leaves<sup>67</sup> and leek<sup>80</sup>), as substrate at a concentration of 10 mM, in the absence and presence of bile salts (either sodium taurodeoxycholate (NaTDC) or sodium deoxycholate (NaDC) at a bile salt/MGDG molar ratio of 1.33<sup>88</sup> or a a bile salt/DGDG molar ratio of 0.25<sup>67</sup>). Lipase activities on tributyrin were measured at pH 8.0 using the pH-stat technique<sup>235</sup>. Values are mean ± SD (n=3) and are expressed in unit (U) per mg of pure enzyme or mg of powder in the case of lyophilized human pancreatic juice (HPJ) and porcine pancreatic extracts (PPE), with 1U = 1 μmole of fatty acid released per minute. rHPLRP2, recombinant human pancreatic lipase-related protein 2; rGPLRP2, recombinant guinea pig pancreatic lipase-related protein; nCEH/ BSSL, native pancreatic carboxyl ester hydrolase or bile salt-stimulated lipase; rCEH/ BSSL, recombinant pancreatic carboxyl ester hydrolase or bile salt-stimulated lipase; nHPL, native human pancreatic lipase; nPPL, native porcine pancreatic lipase; nPPLA2; native porcine pancreatic phospholipase A2. <sup>a</sup>Data from<sup>88</sup>; <sup>b</sup>Data from<sup>67</sup>; <sup>c</sup>Data from<sup>107</sup>; <sup>d</sup>Data from<sup>79</sup>; <sup>e</sup>Data from<sup>80</sup>; <sup>f</sup>Data from<sup>81</sup>; <sup>g</sup>Data from<sup>236</sup>; <sup>h</sup>Data from<sup>185</sup>. Nd, not determined.

| <i>Enzymes</i>                         | <i>C8-MGDG</i>                                       |                          |                            | <i>C8-DGDG</i>          | <i>Long chain MGDG</i>  | <i>Long chain DGDG</i>  | <i>Tributylin</i>                                 |
|--|--|--------------------------|----------------------------|-------------------------|-------------------------|-------------------------|---|
|  | + NaTDC  | + NaDC                   | Without Bile salt          | + NaTDC                 | + NaTDC                 | + NaTDC                 |   |
| rHPLRP2                                | 1786 ± 100 <sup>a</sup>                              | 2800 ± 60 <sup>a</sup>   | 56 ± 2 <sup>a</sup>        | 2976 ± 68 <sup>b</sup>  | 2441 ± 60 <sup>b</sup>  | 1756 ± 208 <sup>b</sup> | 1250 ± 150 <sup>a</sup>                           |
| rGPLRP2                                | 5420 ± 85 <sup>a</sup>                               | 8000 ± 500 <sup>a</sup>  | 700 ± 100 <sup>a</sup>     | 4375 ± 125 <sup>b</sup> | 4167 ± 167 <sup>b</sup> | 3351 ± 170 <sup>b</sup> | 2700 ± 300 <sup>a</sup>                           |
| Human nCEH/ BSSL                       | 230 ± 8 <sup>a</sup>                                 | 240 ± 17 <sup>a</sup>    | 170 ± 2 <sup>a</sup>       | nd                      | nd                      | nd                      | 300 ± 30 <sup>a</sup>                             |
| Human rCEH/BSSL                        | 430 ± 24 <sup>a</sup>                                | 432 ± 62 <sup>a</sup>    | 200 ± 16 <sup>a</sup>      | nd                      | nd                      | nd                      | 470 ± 17 <sup>a</sup>                             |
| Bovine nCEH                            | 31 ± 1 <sup>a</sup>                                  | 56 ± <sup>a</sup>        | 16 ± 2 <sup>a</sup>        | nd                      | nd                      | nd                      | 90 ± 7 <sup>a</sup>                               |
| nHPL                                   | 0  | 0                        | 0                          | nd                      | nd                      | nd                      | 8500 ± 500 <sup>a</sup>                           |
| nPPL                                   | 0  | 0                        | 0                          | nd                      | nd                      | nd                      | 10200 ± 500 <sup>a</sup>                          |
| nPPLA2                                 | 0  | 0                        | 0                          | nd                      | nd                      | nd                      | 0   |
| HPJ                                    | 23 ± 2 <sup>a</sup><br>7.73 ± 0.20 <sup>c</sup>      | 25 ± 1 <sup>a</sup>      | 9 ± 1 <sup>a</sup>         | nd                      | nd                      | nd                      | 964 ± 65 <sup>a</sup><br>231.0 ± 2.7 <sup>c</sup> |
| PPJ                                    | 1.78 ± 0.02 <sup>c</sup>                             | nd                       | nd                         | nd                      | nd                      | nd                      | 231.7 ± 2.9 <sup>c</sup>                          |
| PPE                                    | 0.07 ± 0.02 <sup>a</sup><br>0.31 ± 0.01 <sup>c</sup> | 0.13 ± 0.04 <sub>a</sub> | 0.037 ± 0.005 <sup>a</sup> | nd                      | nd                      | nd                      | 418 ± 7 <sup>a</sup><br>81.7 ± 2.1 <sup>c</sup>   |
| <i>Fusarium solani</i> cutinase        | 984 ± 62 <sup>d</sup>                                | nd                       | nd                         | 300 ± 29 <sup>d</sup>   | nd                      | nd                      | 2596 ± 96 <sup>d</sup>                            |
| <i>Thermomyces lanuginosus</i> lipase  | 450 ± 41 <sup>d</sup>                                | nd                       | nd                         | 672 ± 61 <sup>d</sup>   | nd                      | nd                      | 7834 ± 850 <sup>d</sup>                           |
| <i>Fusarium solani</i> lipase          | 4658 ± 146 <sup>e</sup>                              | nd                       | nd                         | 3785 ± 83 <sup>e</sup>  | 991 ± 85 <sup>e</sup>   | nd                      | 1200 <sup>g</sup>                                 |
| <i>Talaromyces thermophilus</i> lipase | 40500 ± 125 <sup>f</sup>                             | nd                       | nd                         | 9800 ± 125 <sup>f</sup> | nd                      | nd                      | 7300 ± 122 <sup>h</sup>                           |

Table 5: *Effect of the finishing diet on the fatty acid composition (% of total FAs) of intramuscular (IM) and subcutaneous (SC) adipose tissues in horse meat (Galician Mountain breed). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected. Adapted from* <sup>131</sup>

| Finishing diet            | 3 to 5 kg<br>concentrate/day<br>+ hay ad libitum |      | extensive system<br>(free-ranging<br>grass feeding) |             |
|---------------------------|--|------|---|-------------|
|                           |  |      |   |             |
| Finishing (months)        | 3  | 4    | 0   | 0           |
| Age at slaughter (months) | 24   | 24   | 24  | 24          |
| Sex                       | Males and<br>females                             |      | Males and<br>females                                |             |
| Tissue                    | IM   | SC   | IM  | SC          |
| fat (% w/w)               | 0.6  | 63.7 | 0.7   | 60.5        |
| 16:0                      | 10   | 27.9 | 15.8  | 23.4        |
| 18:0                      | 0.61   | 4.6  | 10.7  | 7.4         |
| SFA                       | 10.61  | 32.5 | 26.5  | 30.8        |
| 16:1 n-9                  | 0.7  | 5.5  | 0.8   | 2.8         |
| 18:1 n-9                  | 6.3  | 25.1 | 7.7   | 27.5        |
| MUFA                      | 7  | 30.6 | 8.5   | 30.3        |
| 18:2 n-6                  | 32.4   | 15.9 | 29.9  | 7.6         |
| 20:2 n-6                  |  |      |   |             |
| 20:3 n-6                  |  |      |   |             |
| 20:4 n-6                  | 6.7  | ND   | 5.8   | ND          |
| n-6                       | 39.1   | 15   | 35.7  | 7.6         |
| <u>18:3 n-3 (ALA)</u>     | 0.9  | 7.6  | 4.5   | <u>24.3</u> |
| 20:3 n-3                  |  |      |   |             |
| 20:5 n-3                  | 0.9  | ND   | 1.7   | ND          |
| 22:5 n-3                  | ND   | 0.2  | 3.3   | 0.2         |
| 22:6 n-3                  | 0.5  | ND   | 0.8   | ND          |
| n-3                       | 2.3  | 7.8  | 10.3  | 24.5        |
| PUFA                      | 41.4   | 23.7 | 46  | 32.1        |
| n-6 to n-3 ratio          | 17.32  | 2.28 | 3.79  | 0.55        |
| Reference                 | 133  | 133  | 133   | 133         |

Table 6: Fatty acid composition (mg/g DW) of total body lipids in zebrafish fed with either a control fish diet or fishmeal reduction up to a level of 50 % w/w of spinach chloroplast-rich fraction (CRF) or spinach leave powder (SLP). <sup>a</sup> significantly higher versus controls; <sup>b</sup> significantly higher in controls. Data from <sup>145</sup>.

| <b>Fatty acids</b> | <b>Control</b>          | <b>CRF 50</b>           | <b>SLP50</b>            |
|--------------------|-------------------------|-------------------------|-------------------------|
| 14:0               | 1.9 ± 0.0               | 1.8 ± 0.0               | 1.7 ± 0.0               |
| 16:0               | 21.5 ± 0.3              | 28.9 ± 0.1 <sup>a</sup> | 26.2 ± 0.9 <sup>a</sup> |
| 16:1 n-7           | 2.4 ± 0.1               | 2.6 ± 0.0               | 2.2 ± 0.2               |
| 16:3 n-3           | 0.03 ± 0.0              | 1.1 ± 0.0 <sup>a</sup>  | 0.4 ± 0.0 <sup>a</sup>  |
| 18:0               | 3.0 ± 0.0               | 4.4 ± 0.2               | 4.7 ± 0.3               |
| 18:1 n-9           | 20.2 ± 1.4 <sup>b</sup> | 19.7 ± 0.4              | 17.6 ± 0.8              |
| 18:2 n-6           | 15.0 ± 0.5 <sup>b</sup> | 13.7 ± 0.0              | 12.8 ± 0.8              |
| 18:3 n-3 (ALA)     | 3.0 ± 0.0               | 7.3 ± 0.0 <sup>a</sup>  | 4.1 ± 0.2 <sup>a</sup>  |
| 20:4 n-6           | 0.5 ± 0.0               | 0.5 ± 0.0               | 0.6 ± 0.0               |
| 20:5 n-3 (EPA)     | 1.5 ± 0.1               | 1.4 ± 0.0               | 1.4 ± 0.0               |
| 22:6 n-3 (DHA)     | 6.0 ± 0.1               | 6.2 ± 0.1               | 5.6 ± 0.3               |
| Σn-6               | 15.5 <sup>b</sup>       | 14.2                    | 13.4                    |
| Σn-3               | 10.5                    | 16 <sup>a</sup>         | 11.4                    |
| n-6 to n-3 ratio   | 1.47 <sup>b</sup>       | 0.89                    | 1.17                    |



Table 7: Omega-3 fatty acid composition (% of total FA; mean value  $\pm$  SD) of the fat extracted from farmed food fishes. Adapted from <sup>157</sup>.

| <i>Fatty acids</i> | <i>Grass carp</i> | <i>Bighead carp</i> | <i>Siberian sturgeon</i> | <i>Wels catfish</i> |
|--------------------|-------------------|---------------------|--------------------------|---------------------|
| 18:3 n-3 (ALA)     | 7.91 $\pm$ 3.60   | 3.52 $\pm$ 0.77     | 3.36 $\pm$ 0.32          | 4.19 $\pm$ 0.48     |
| 20:5 n-3 (EPA)     | 1.10 $\pm$ 0.50   | 2.92 $\pm$ 0.80     | 2.38 $\pm$ 0.25          | 1.63 $\pm$ 0.42     |
| 22:5 n-3 (DPA)     | 0.86 $\pm$ 0.30   | 0.56 $\pm$ 0.24     | 0.79 $\pm$ 0.13          | 0.85 $\pm$ 0.24     |
| 22:6 n-3           | 1.08 $\pm$ 0.35   | 2.29 $\pm$ 0.66     | 3.7 $\pm$ 0.37           | 3.01 $\pm$ 0.81     |