Lipases in wheat flour bread making: importance of an appropriate balance between wheat endogenous lipids and their enzymatically released hydrolysis products

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Abbreviations[1]

BM, bread making; DAG, diacylglycerols; db, dry matter base; DGDG, digalactosyldiacylglycerols; DGMG, digalactosylmonoacylglycerols; FFA, free fatty acids; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholines; LV, loaf volume; MAG, monoacylglycerols; MGDG, monogalactosyldiacylglycerols; MGMG, monogalactosylmonoacyl-glycerols; NALPE, N-acyl lysophosphatidylethanolamines; NAPE, N-acyl phosphatidylethanolamines; PC, phosphatidylcholines; RT, room temperature; TAG, triacylglycerols; WSB, water-saturated butan-1-ol

[1] Although most of the acronyms are defined as plural, they can be read as singular when that is more appropriate

Abstract

Lipids are only minor wheat flour constituents but play major roles in bread making (BM). Here, the importance of a well-balanced lipid population in BM was studied by applying a lipase from *Fusarium oxysporum* in the process. Monogalactosyldiacylglycerols and N-acyl phosphatidylethanolamines were the most accessible lipase substrates. Hydrolysis thereof into their corresponding lysolipids was largely if not entirely responsible for loaf volume increases upon lipase application. Degradation of endogenously present lipids and enzymatically released lysolipids caused loaf volume to decrease, confirming that an appropriate balance between different types of lipids is crucial in BM. For optimal dough gas cell stability, the level of lipids promoting lamellar mesophases and, thus, liquid condensed monolayers needs to be maximal while maintaining an appropriate balance between lipids promoting hexagonal I phases, non-polar lipids and lipids promoting hexagonal II or cubic phases.

Key words

Lipase, oil body, Lipopan F, loaf volume, gas cell stabilization, mesophase, monolayer

Chemical compounds studied in this article

Hexane (PubChem CID: 8058); butan-1-ol (PubChem CID: 263); *p*-nitrophenyl palmitate (PubChem CID: 73891); *p*-nitrophenol (PubChem CID: 980); isooctane (PubChem CID: 10907); cholesterol (PubChem CID: 5997)

1 Introduction

Wheat flour contains 2.0 – 3.0% lipids, which are classified as non-starch (60 – 70%) or starch (30 – 40%) lipids depending on their location. Traditionally, non-starch lipids are further subdivided into free (*ca.* 60%) and bound (*ca.* 40%) lipids based on their extractability with different organic solvents at room temperature (RT). Free lipids are extracted with non-polar solvents (*e.g.* hexane, ether or petroleum ether) and bound lipids with polar solvents [*e.g.* water-saturated butan-1-ol (WSB)] after removing the free lipids. Starch lipids are located inside the granular starch structure. They are typically extracted with a polar solvent [*e.g.* WSB or isopropanol:water (90:10)] at 90 – 100°C after first removing all non-starch lipids. Non-starch lipids consist of 51 – 63% non-polar lipids, 22 – 27% galactolipids and 13 – 23% phospholipids, with as most common lipids triacylglycerols (TAG, 21 – 47%), digalactosyldiacylglycerols (DGDG, 13 – 17%), monogalactosyldiacylglycerols (MGDG, 5 – 6%), N-acyl phosphatidylethanolamines (NAPE, 4 – 5%) and phosphatidylcholines (PC, 4 – 6%). The free lipid fraction contains both non-polar lipids (75%) and polar lipids (25%, *i.e.* galactolipids and phospholipids) whereas the bound lipid fraction essentially consists of polar lipids. The major fatty acids in non-starch lipids are linoleic (50 – 65%), palmitic (19 – 26%) and oleic (10 – 21%) acids. Starch lipids are mostly phospholipids (85 – 91%) of which about 85% are lysophosphatidylcholines (LPC), and contain linoleic (38 – 48%), palmitic (39 – 45%) and oleic (8 – 12%) acids (Chung, Ohm, Ram, Park, & Howitt, 2009; Morrison, 1978; Morrison, Mann, Soon, & Coventry, 1975; Pareyt, Finnie, Putseys, & Delcour, 2011).

Although lipids are only minor wheat flour constituents, they play major roles in bread making (BM) (Chung, et al., 2009). In 1978, Chung, Pomeranz and Finney reviewed the role of wheat flour lipids in the process. They concluded that much information was available on this topic, but that it was fragmentary and sometimes contradictory. According to the authors, factors that made sustained and consistent progress difficult included the complexity of lipid level and composition, variations in lipids of wheat cultivars from various locations, changes during extraction and storage of lipids, effects of lipid extraction

on functional properties of wheat flour components and multiple component interactions of flour lipids, shortening and surfactants. As a result, they stated that there was little likelihood that a unified theory on the role of wheat lipids in the production of baked goods could be developed and concluded with the following wording: *"Consequently, we will learn increasingly more about the role of lipids, but we will be frustrated by the complexity of the effects."*. After another 30 years of research into lipids in BM, which was reviewed a number of times (Carr, Daniels, & Frazier, 1992; Chung, et al., 2009; MacRitchie, 1981; Pareyt, et al., 2011), this statement unfortunately has withstood the test of time.

Nonetheless, fractionation-reconstitution experiments have delivered insights in the effects that wheat flour lipids have on bread quality. It is generally accepted that the flour non-polar lipid fraction depresses whereas the polar lipid fraction increases loaf volume (LV) (Daftary, Pomeranz, Shogren, & Finney, 1968; De Stefanis & Ponte, 1976; MacRitchie & Gras, 1973). The flour non-polar lipid fraction contains not only pure non-polar lipids but also lipids of intermediate polarity such as free fatty acids (FFA) (Chung, et al., 2009). They are the main responsible for the negative effects of the non-polar lipid fraction while the more non-polar compounds such as TAG have little effect on the volume of bread made from defatted flour. Linoleic and myristic acids have a negative while palmitic acid has little effect on LV. Flour polar galactolipids contribute more to BM quality than do phospholipids (Daftary, et al., 1968; De Stefanis, et al., 1976; Sroan & MacRitchie, 2009). Most studies, including the ones cited above, exclusively deal with the role of flour non-starch lipids since starch lipids, although they may be important in staling, do not appear to affect LV as they occur inside starch granules and are therefore not available in the continuous dough matrix (MacRitchie, 1981; MacRitchie, et al., 1973; Morrison, 1978).

More recently, lipases have been used to study the role of wheat flour lipids in BM (Gerits, Pareyt, & Delcour, 2014; Gerits, Pareyt, Masure, & Delcour, 2015; Schaffarczyk, Østdal, & Koehler, 2014; Schaffarczyk, Østdal, Matheis, & Koehler, 2016). Such enzymes catalyze hydrolysis of the ester bond(s) of glycero(galacto- or phospho-)lipids and thereby modify the wheat endogenous lipid population. In BM, 60

– 70% of the wheat flour lipids (*i.e.* the non-starch lipids) can act as a substrate for lipases. The starch lipids are essentially unavailable due to their location inside the granular structure (De Maria, Vind, Oxenbøll, Svendsen, & Patkar, 2007). Lipases improve dough processing as well as overall bread quality by increasing dough stability, maximum resistance to extension and hardness, decreasing dough stickiness, increasing specific LV, improving crumb softness and structure and delaying retrogradation during storage (Aravindan, Anbumathi, & Viruthagiri, 2007; Colakoglu & Özkaya, 2012; De Maria, et al., 2007; Frauenlob, Scharl, D'Amico, & Schoenlechner, 2018; Gerits, et al., 2014; Gerits, et al., 2015; Moayedallaie, Mirzaei, & Paterson, 2010). It has also been suggested that lipases can partially or completely replace surfactants in BM as they *in situ* generate surfactant-like molecules by hydrolyzing wheat endogenous lipids (Aravindan, et al., 2007; Colakoglu, et al., 2012; De Maria, et al., 2007; Moayedallaie, et al., 2010). Their effects are generally described in terms of producing more polar lipids by hydrolyzing one or more fatty acids from non-polar and/or polar lipids and, thus, improving surface activity of the lipid population (Colakoglu, et al., 2012; Gerits, et al., 2014; Moayedallaie, et al., 2010; Schaffarczyk, et al., 2014). However, not all lipases are equally effective at improving bread quality and some even diminish LV (De Maria, et al., 2007). Analysis of lipase-treated wheat lipids revealed that hydrolysis of galactolipids and phospholipids is most important when the goal is to increase LV (De Maria, et al., 2007; Gerits, et al., 2014; Schaffarczyk, et al., 2014). An optimal BM lipase preferably hydrolyzes MGDG and NAPE while having only moderate activity towards DGDG. Indeed, DGDG and its products [digalactosylmonoacylglycerols (DGMG)] act in a synergistic manner (Schaffarczyk, et al., 2016). Finally, an overdose of lipase and thus extensive lipid hydrolysis causes LV to decrease. This denotes the importance of reaching an optimal lipid composition (Gerits, et al., 2014; Schaffarczyk, et al., 2014).

Based on the above, we hypothesize that an appropriate balance between endogenously present lipids, their corresponding lysolipids and FFA is crucial in wheat flour BM. The aim of this study was to examine the importance of a well-balanced lipid population in BM and to propose a mechanism which

fully explains the improving effects which BM lipases exert. Hereto, a successful BM lipase was applied at different levels in laboratory-scale BM and its impact on bread LV and dough lipid population was evaluated. Lipase application allows selective modification of the wheat endogenous lipids without altering other flour constituents and therefore identifying the functionality and technological effects of different lipids, either endogenously present or enzymatically released. Using different lipase levels permitted pinpointing the optimal balance of (enzymatically modified) lipids in BM. The obtained findings expand the current state-of-the-art in the form of a unified theory on the role of wheat endogenous lipids (and their enzymatically released hydrolysis products) in BM. Because starch lipids do not influence fresh bread properties (MacRitchie, 1981; MacRitchie, et al., 1973; Morrison, 1978) they were not considered in this study and the term "lipids" in the remainder of the text therefore refers to non-starch lipids only.

2 Materials and methods

2.1 Materials

Grains (± 25 g) from soft wheat cultivar Alpowa were kindly donated by Dr. C.F. Morris (Western Wheat Quality Laboratory, USDA-ARS, Pullman, WA, USA), cultivated in greenhouses (Faculty of Bioscience Engineering, KU Leuven, Leuven, Belgium) and further multiplied on small plots (Gembloux Agro-Bio Tech, Gembloux, Belgium). Grains were first conditioned to a moisture level of 16.0% before milling into straight grade flour with a Bühler MLU-202 laboratory mill (Uzwil, Switzerland) as described earlier (Delcour, Vanhamel, & De Geest, 1989). Milling yield was 68.6% and flour moisture level, determined with AACCI Approved Method 44-15.02, was 13.6%. Flour ash and protein levels (N x 5.7) were 0.76% (± 0.02%) and 10.6% (± 0.0%) on dry matter base (db), respectively, as determined in triplicate with AACCI Approved Method 08-01.01 and an adaptation of the AOAC Official Method to an automated Dumas protein analysis system (VarioMax Cube N, Elementar, Hanau, Germany), respectively. Flour free

and bound lipid levels (on db) were 0.60% (± 0.00%) and 0.69% (± 0.03%), respectively, as determined in triplicate as described in §2.4.

The BM lipase from *Fusarium oxysporum* expressed in *Aspergillus oryzae* (Lipopan F; Novozymes, Bagsværd, Denmark) was kindly donated by Puratos (Groot-Bijgaarden, Belgium). This lipase is supplied as an off-white granulate and contains next to the enzyme, wheat flour, sodium chloride and dextrin. Its recommended use level in baking applications is up to 50 ppm (Novozymes, 2001). It had a lipase activity towards *p*-nitrophenyl palmitate of 2.8 µmol *p*-nitrophenol/(min.mg) which was determined as in Melis, Pauly, Gerits, Pareyt and Delcour (2017) but by preparing a lipase extract (as described in §2.3) because the Lipopan F granulates are not soluble in water. The lipase contained negligible amylase, peptidase and xylanase side activities (results not shown).

Sugar, salt and fresh compressed baker's yeast (Koningsgist, AB Mauri, Dordrecht, The Netherlands) were purchased at a local super market.

2.2 Experimental setup

First, changes in LV as a function of lipase level for bread prepared from a recipe containing lipase from *F. oxysporum* were evaluated. It was hypothesized that enzymatically altering the endogenous lipid composition causes a change in bread LV with the nature (i.e. increase or decrease) and extent depending on the extent of enzymatic lipid hydrolysis. From the obtained plot of LV as a function of lipase level, three lipase levels were carefully selected for further analyses. These corresponded with a suboptimal increase, an optimal increase and a significant decrease in LV. Next, the lipid populations in both freshly mixed and fermented doughs containing the lipase in the selected levels were studied. The lipid populations in control doughs lacking lipase were also measured to have appropriate controls. Changes in bread LV and dough lipid population induced by applying the chosen lipase levels in bread making were related to each

169 other to increase insight in the importance of an appropriate balance between different wheat
170 endogenous lipids and their enzymatically released hydrolysis products.
171
172 2.3 Dough and bread making
173 Bread was prepared on 10 g scale according to the straight-dough procedure of Shogren and Finney
174 (1984) but without shortening, malted barley, ascorbic acid or potassium bromate. First, 10.00 g flour
175 (14.0% moisture base) was mixed with 5.7 mL deionized water, 0.53 g yeast, 0.15 g salt and 0.60 g sugar
176 for 4.0 min in a 10 g pin mixer (National Manufacturing, Lincoln, NE, USA). Water level and optimal mixing
177 time were based on Farinograph (Farinograph-E, Brabender, Duisburg, Germany) and mixograph (National
178 Manufacturing) analyses according to AACCI Approved Methods 54-21.02 and 54-40.02, respectively.
179 To avoid weighing errors and because the Lipopan F granulates are not soluble in water, a lipase
180 extract was prepared for including lipase in the recipe. Briefly, 100 mg Lipopan F and 5.0 mL deionized
181 water were shaken (30 min, 150 rpm, RT) in a screw cap test tube. Following centrifugation (5 min, 4,000
182 g, RT) the supernatant was filtered. Per mL, it contained the material extracted from 20 mg of lipase
183 granulates. The supernatant was diluted with deionized water in extents ranging from 3.8 to 2,280 times.
184 Dough was then prepared by adding 5.7 mL diluted lipase solution instead of deionized water. By way of
185 example, when 5.7 mL of 3.8 times diluted lipase solution was added in dough making, dough contained
186 the material extracted from 30 mg of lipase granulates. In this example, dough contained 3,000 mg/kg
187 flour (i.e. ppm) lipase. As such, lipase was included in the recipe in levels ranging from 5 to 3,000 ppm,
188 where the amount of lipase applied in BM (*e.g.* x mg) corresponds to the material extracted from an
189 equivalent amount (*e.g.* x mg) of lipase granulates.
190 After mixing, dough pieces were shaped and placed in a fermentation cabinet (National
191 Manufacturing) for 90 min (30 °C, 90% relative humidity) with intermediate punching at 52 and 77 min
192 and final punching at 90 min. Following molding and proofing (36 min at 30 °C, 90% relative humidity),

dough pieces were baked at 232 °C for 13 min in a rotary oven (National Manufacturing). Bread loaves were weighed immediately after removal from the oven. Their volumes were measured after cooling for 120 min with a VolScan Profiler (Stable Micro Systems, Godalming, Surrey, UK) with a rotation speed of 1.0 rps and a vertical step size of 1 mm. Breads from control and lipase containing recipes were prepared in duplicate.

For lipid extraction and analysis, both freshly mixed (not fermented) and fermented (126 min) dough samples were prepared and immediately frozen with liquid nitrogen, freeze dried, and ground with a laboratory mill (model A10, IKA-Werke KG, Staufen, Germany) to pass a 250 µm sieve (Retsch, Haan, Germany). Doughs from control and lipase containing recipes were prepared in triplicate so that per treatment, three dough samples were obtained for investigating the dough lipid population. These processed dough samples were stored at -18 °C for up to one month until further analysis.

2.4 Lipid extraction

Free and bound lipids were extracted from wheat flour, freshly mixed dough and fermented dough as in Melis et al. (2017) but by blending 1.00 g sample (14.0% moisture base) with 28 g sand (acid washed; Sigma-Aldrich, Bornem, Belgium) prior to extraction. Extraction from wheat flour was in triplicate. Single extractions were performed on dough samples since three dough samples were obtained per treatment yielding three dough lipid extracts per treatment (§2.3). The obtained dry lipid extracts of freshly mixed and fermented dough samples were stored under nitrogen atmosphere in amber-colored vials at -80 °C until further analysis.

2.5 Lipid population analysis

To the dry lipid extracts of freshly mixed and fermented dough samples (obtained in §2.4) cholesterol (Larodan, Solna, Sweden) was added as internal standard (5.0 mg for free and 2.5 mg for

bound lipid extracts) to ensure method reproducibility and to allow for proper comparison between different samples. The lipids were then dissolved in 1.0 mL isooctane [high-performance liquid chromatography (HPLC) grade; Merck KGaA, Darmstadt, Germany], separated with HPLC and detected with evaporative light scattering as in Melis et al. (2017). An aliquot of each sample (1.0 µL) was injected. Single lipid analyses were performed on dry lipid extracts of dough samples since three dough lipid extracts per treatment were obtained (§2.4). Identification of lipid classes was based on previous work of Gerits, Pareyt and Delcour (2013). Lipid levels are expressed as the areas under the curve relative to that of the internal standard.

2.6 Statistical analysis

Statistical analysis was performed using JMP 14 software (SAS Institute, Cary, NC, USA). For lipid levels, it was verified whether mean values differed significantly using one-way ANOVA. In addition, when lipid levels were significantly different ($p < 0.05$), means were further compared using the post-hoc Tukey-Kramer test with a significance level (α) of 0.05.

3 Results

3.1 Bread loaf volume

The flour used in this study produced loaves of acceptable quality. Figure 1 shows the change in specific LV as a function of lipase level. LV did not change when low levels of 5 to 10 ppm lipase were included, whereas it increased by approximately 11% and 15% when the lipase was used in levels of 20 to 100 ppm and 200 to 700 ppm, respectively. From 700 ppm onwards, increasing lipase levels resulted in a reduced LV, with loaves 11% smaller than control breads (without lipase) when 3,000 ppm was applied. Plotting the change in specific LV as a function of lipase level for the investigated lipase thus revealed ranges of levels where the lipase performed suboptimally (20-100 ppm) and optimally (200-700 ppm) in

terms of increasing LV. Applying too little lipase (< 10 ppm) had no effect on LV whereas overdosing (> 700 ppm) resulted in significantly smaller loaves.

3.2 Dough lipid population

The lipids extracted with hexane (free lipids, see §1) from freshly mixed and fermented dough prepared with and without different levels of the BM lipase consisted almost exclusively of TAG and FFA, of which the levels are shown in Figure 2. Free TAG levels in mixed and fermented dough dropped significantly upon addition of 20 and again upon addition of 200 ppm lipase, demonstrating hydrolysis of free TAG proportional to lipase concentration. Strangely enough, hydrolysis of free TAG was not proportional to lipase reaction time, as no differences in free TAG levels were detected between freshly mixed and fermented dough containing lipase. We had expected (further) hydrolysis of free TAG during fermentation as

(i) they were depleted neither in dough containing 20 nor in that containing 200 ppm lipase, and

(ii) the lipase had sufficient time (126 min, vs. only 4 min mixing) to affect the dough lipid population in fairly optimal conditions (30 °C, pH 5.0-6.0).

Nonetheless, free TAG were only hydrolyzed during mixing while (further) hydrolysis during fermentation did not occur. Increased levels of free FFA were observed in mixed dough containing lipase, with a maximum level in mixed dough with 3,000 ppm lipase. In fermented dough, however, free FFA levels in dough containing 3,000 ppm lipase were lower than in dough containing 200 ppm lipase. The results indicate a shift in the extractability of FFA. This has been observed before by Melis et al. (2017) for FFA in gluten isolated according to a dough-batter wheat flour separation process. They assumed that at least some of the FFA formed during wheat flour separation with intensive enzymatic hydrolysis of (non-polar) lipids are present in their carboxylate form and are therefore not extractable with non-polar solvents like hexane.

The lipids extracted with WSB after removing the free lipids (bound lipids, see §1) from freshly mixed and fermented dough made with and without different levels of lipase contained next to TAG and FFA also monoacylglycerols (MAG), galactolipids and phospholipids, the levels of which are shown in Figure 3. The level of bound TAG in both freshly mixed and fermented dough containing 20 ppm lipase equaled that in dough without added lipase. Hence, bound TAG were not hydrolyzed at a lipase level of 20 ppm. When 200 and 3,000 ppm lipase levels were applied, bound TAG were hydrolyzed and, much as noted for free TAG, hydrolysis occurred only during mixing and not during fermentation. MAG accumulated in dough containing 200 ppm lipase implying that hydrolysis of diacylglycerols (DAG) occurred faster than that of MAG. In mixed dough containing 3,000 ppm lipase, MAG accumulation levelled off and in fermented dough containing 3,000 ppm lipase, MAG levels significantly dropped. At this point in time, DAG were probably more or less depleted so that less MAG were produced than hydrolyzed. Bound FFA levels in mixed and fermented dough were unaffected by a 20 ppm lipase dosage but increased proportionally with lipase concentration and reaction time.

With regard to galactolipids, results demonstrate that the lipase hydrolyzed MGDG, the intensity of hydrolysis being proportional to both lipase concentration and reaction time. While 20 ppm lipase dosage did not impact MGDG levels in mixed dough, it did lead to a significant drop of its level during fermentation. Both in mixed and fermented dough, hydrolysis was more pronounced at lipase levels of 200 and especially 3,000 ppm. Concomitant increases in levels of monogalactosylmonoacylglycerols (MGMG) in mixed and fermented dough containing 20 or 200 ppm lipase and in mixed dough containing 3,000 ppm lipase were observed. In fermented dough containing 3,000 ppm lipase, MGMG levels were significantly lower. The lysolipids (MGMG) produced by hydrolysis of wheat endogenous lipids (MGDG) were further hydrolyzed into FFA and their polar backbones. Comparable phenomena were observed for DGDG and DGMG, but with slightly different responses to lipase concentration. Indeed, hydrolysis of DGDG and concomitant accumulation of DGMG was not observed at 20 ppm lipase. Again, in fermented

289 dough containing 3,000 ppm lipase, the lysolipids (DGMG) produced from DGDG were further hydrolyzed
290 as demonstrated by the significant drop in DGMG levels.

291 The way the hydrolysis of the phospholipid NAPE depended on lipase concentration and reaction
292 time was comparable to that of MGDG. Indeed, NAPE levels significantly dropped as a result of
293 fermentation in dough containing 20 ppm lipase and hydrolysis was more pronounced at higher lipase
294 levels. Concomitant increases in levels of N-acyl lysophosphatidylethanolamines (NALPE) were observed
295 in dough containing 20 ppm but not in that containing 200 or 3,000 ppm lipase. In fermented dough
296 containing 3,000 ppm lipase, NALPE were even no longer detected. Apparently, NALPE are more easily
297 hydrolyzed into FFA and polar backbones than MGMG or DGMG. PC were, in comparison with the other
298 investigated polar lipids, hydrolyzed relatively slowly and much as TAG only during mixing as their levels
299 in mixed and fermented dough were very comparable if not identical. No concomitant accumulation of
300 LPC was observed. Instead, LPC levels in dough containing 200 or 3,000 ppm lipase dropped significantly
301 during fermentation, pointing out further hydrolysis of these lysolipids.

302

303 **4 Discussion**

304 4.1 Degradation of triacylglycerols and phosphatidylcholines by the lipase in bread making

305 Investigation of the lipid population of freshly mixed and fermented dough containing variable
306 levels of lipase (Figures 2 and 3) revealed that hydrolysis of wheat endogenous lipids was proportional to
307 both lipase concentration and reaction time, except for that of TAG and PC. Strangely enough, these lipids
308 were only hydrolyzed during mixing while further hydrolysis during fermentation did not occur.

309 TAG in plant seeds are present in discrete oil bodies or spherosomes of 0.2 to 2.5 µm (Huang, 1992;
310 Tzen & Huang, 1992). In wheat, these oil bodies occur in large numbers in the scutellum and aleurone and
311 in lesser quantities in the embryo, subaleurone and starchy endosperm (Hargin & Morrison, 1980). A
312 typical oil body has a core of TAG surrounded by a monolayer of phospholipids in which oleosins, *i.e.*

alkaline proteins of low molecular mass (15 to 26 kDa), are embedded and from which they protrude (Huang, 1992; Tzen, et al., 1992). PC account for nearly two-thirds of the total phospholipids in oil bodies of wheat, the remainder being equal portions of phosphatidylethanolamine and phosphatidylinositol (Jelsema, Morré, Ruddat, & Turner, 1977). Oleosin proteins consist of an hydrophobic stalk embedded in the hydrophobic acyl moieties of phospholipids and the TAG core and an amphipathic domain protruding from and covering the oil body. They shield the phospholipid shell so that neither phospholipase A_2 nor phospholipase C can act on the phospholipids and affect the stability of the oil bodies, as demonstrated by Tzen et al. (1992).

Possibly, TAG and PC are only enzymatically hydrolyzed during mixing but not during fermentation due to their structural organization in oil bodies. Based on the above, it is reasonable to assume that oil bodies resist degradation by lipase in BM. Oil bodies assume a spherical shape except when pressed into irregular shapes by other cell structures in mature seeds (Huang, 1992). We hypothesize that dough mixing deforms the spherical oil bodies in wheat flour due to the high-shear conditions. Upon deformation, their surface area and, thus, the distance between adjacent oleosin molecules likely increases, resulting in incomplete shielding of the phospholipid layer underneath the protruding oleosins which then allows degradation of oil bodies by lipases. It is unclear how (partially) degraded oil bodies resist further enzymatic degradation.

4.2 Most accessible lipase substrates in bread making

Studying the lipids present in freshly mixed and fermented dough containing variable levels of lipase allowed identifying its most accessible substrates. Lipid substrates hydrolyzed at the lowest lipase levels and reaction times are regarded to be the most accessible substrates. Of all investigated lipids, free TAG were hydrolyzed fastest as they were the only lipids of which the level significantly dropped when mixing dough containing 20 ppm lipase (Figure 2). However, because they are unavailable for hydrolysis during

fermentation (§4.1), free TAG can hardly be called the most accessible substrates of the investigated lipase. In fermented dough, the only lipids of which the levels significantly had dropped upon addition of 20 ppm lipase were MGDG and NAPE, with concomitant increases in the levels of their corresponding lysolipids, MGMG and NALPE (Figure 3). Hence, in BM without use of surfactants, oils or fats, MGDG and NAPE are the most accessible substrates of the lipase used in the present study. Schaffarczyk et al. (2016) earlier stated that an optimal BM lipase preferably hydrolyzes MGDG and NAPE, which is confirmed by our findings. These substrates are indeed preferentially hydrolyzed by the successful BM lipase used.

4.3 Relating dough lipid population to bread loaf volume

Lipase application in BM allows selectively modifying dough lipids without altering other constituents. Changes in bread LV upon lipase use are therefore exclusively brought about by modifying the lipid population. Hence, lipases are excellent tools to investigate the role of lipids in BM. Currently, wheat endogenous lipids are believed to mainly affect BM by being present at gas/liquid interfaces in dough and thereby contributing directly to gas cell stabilization (§4.4). As interfacial gas cell stabilization in dough is most important during the later stages of fermentation and initial stages of baking, *i.e.* when gas cells are no longer completely surrounded by the continuous gluten network (Gan, Ellis, & Schofield, 1995; Sroan, et al., 2009), it seems logical that the lipid population in fermented rather than in mixed dough is related to bread LV. The here obtained results support this reasoning as it is highly unlikely that a LV increase of 11% upon inclusion of 20 ppm lipase in BM (Figure 1) would be explained by a partial hydrolysis of free TAG, the only lipid modification detected in mixed dough with 20 ppm lipase (Figures 2 and 3).

During fermentation of dough containing 20 ppm lipase, not only the level of free TAG but also those of MGDG and NAPE dropped significantly, with corresponding increases in the levels of free FFA, MGMG and NALPE. In contrast, the levels of other investigated lipids remained unchanged. Hence,

hydrolysis of the most accessible substrates of the lipase (MGDG and NAPE, see §4.2) into their corresponding lysolipids (MGMG and NALPE) was responsible for LV increases of 11%.

When using a lipase level of 200 ppm, LV increased by another 3% so that loaves 15% larger than control breads were obtained (Figure 1). At this lipase level, almost all investigated lipids were affected during mixing and/or fermentation, resulting in significant increases in FFA levels. TAG and subsequent DAG hydrolysis (which was not detectable) resulted in accumulation of MAG, hydrolysis of the galactolipids (MGDG and DGDG) in accumulation of the corresponding lysolipids (MGMG and DGMG), whereas the lysolipids derived from phospholipid hydrolysis (NALPE and LPC) were further hydrolyzed. Phospholipids containing only one esterified fatty acid are thus more easily hydrolyzed than galactolipids with one esterified fatty acid. As almost all investigated lipids were significantly affected, the obtained results do not allow identifying the lipid modification responsible for a small additional LV increase upon increasing lipase level from 20 to 200 ppm. However, we believe that the additional volume increase is caused by more intense hydrolysis of the most accessible substrates (MGDG and NAPE) rather than by modifications of other lipids since less intense degradation of the most accessible substrates alone (at 20 ppm lipase) had a substantial positive effect on LV.

Overdosing the lipase by including 3,000 ppm in the recipe significantly reduced bread LV (Figure 1). At this lipase level, not only the endogenously present but also lysolipids produced through lipase action are (further) hydrolyzed until an important part of them is (almost) no longer detectable in fermented dough (Figure 3). Most lipids except FFA are thus removed, with a clear negative impact on LV. Since removing wheat endogenous lipids (including FFA) by using defatted flour in BM has either no significant impact on or even improves bread LV (MacRitchie, et al., 1973; Sroan, et al., 2009), this confirms the unfavorable role of FFA in the process. Negative functionalities have been ascribed to unsaturated FFA in BM, whereas saturated FFA have been found to play a neutral role (De Stefanis, et al., 1976; Sroan, et al., 2009). Wheat non-starch lipids contain about 78% unsaturated and thus only about 22% saturated

fatty acids (Chung, et al., 2009) so that severe enzymatic hydrolysis of wheat lipids, such as after fermentation of dough containing 3,000 ppm of the investigated lipase (Figures 2 and 3), undoubtedly releases vast amounts of unsaturated FFA. Overall, these results demonstrate that an appropriate balance between endogenously present lipids, their corresponding lysolipids and FFA is of major importance in BM.

4.4 Mechanism whereby wheat endogenous lipids and their enzymatically released hydrolysis products affect loaf volume

The influence of wheat endogenous lipids on bread LV and crumb structure is related to the stability of the gas cells in bread dough (MacRitchie, 1981; MacRitchie, et al., 1973). During early fermentation, gas cells are embedded in and supported by the viscoelastic gluten network. This network fails to completely surround the gas cells at advanced stages of fermentation and early stages of baking. From that moment onwards, a thin liquid film surrounding the gas cells and containing surface active proteins and (polar) lipids is thought to take over gas cell stabilization (Gan, et al., 1995; Sroan, et al., 2009). Lipids have been assumed to affect gas cell stability

(i) indirectly by interacting with gluten and thereby strengthening the gluten network, and

(ii) directly by their presence as surface active agents in the liquid film surrounding gas cells.

During dough mixing, part of the non-polar and most of the polar free lipids of wheat flour become bound, mainly to gluten, a phenomenon referred to as “lipid binding” (Carr, et al., 1992; Chung, et al., 2009; Olcott & Mecham, 1947). In BM, because of the amphiphilic nature of polar lipids, lipid binding strengthens the viscoelastic gluten network either by decreasing electrostatic repulsion between gluten polymers (Köhler, 2001) or by effectively bridging starch and gluten components (Pomeranz & Chung, 1978).

408 The above indirect contribution of lipids to gas cell stability is, however, arguable. Mainly as a result
409 of the work by MacRitchie and coworkers (MacRitchie, 1981; MacRitchie, et al., 1973; Sroan, et al., 2009),
410 the influence of wheat endogenous lipids on LV and crumb structure is currently believed to be exclusively
411 related to their direct stabilization of dough gas/liquid interfaces. MacRitchie et al. already in 1973
412 presented evidence that lipids do not contribute significantly to the bulk rheological properties of doughs
413 and in 2009, Sroan et al. demonstrated that lipids at their natural flour levels do not affect dough rheology
414 as measured by bubble inflation. More recent findings by Gerits et al. (2015) support this view. The
415 authors found that dough rheology (as examined by Kieffer rig extension tests) is not affected by lipase
416 use and concluded that it is only influenced by the lipid population readily available at the start of mixing
417 and not by the lipase reaction products released during fermentation. The impact of lipases, and therefore
418 of enzymatically modified lipids, on bread LV and crumb structure in their view is thus related to direct
419 interfacial stabilization of gas cells in bread dough.
420 The surface activity of lipids and thus their ability to directly stabilize gas cells in bread dough is
421 determined by the type of monolayer they form at gas/liquid interfaces as well as by their behavior in the
422 dough aqueous phase (Selmair, 2010). An overview of the possible orientations of lipids at gas/liquid
423 interfaces and in aqueous phases in wheat flour bread making is presented in Figure 4. By applying the
424 lipase used in the present work in bread making, the endogenous lipid population is modified and the
425 balance between lipids promoting different mesophases and monolayers is controlled by the applied level
426 (Figure 2 and Figure 3).
427 Applying 20 ppm lipase caused the level of lipids promoting hexagonal II or cubic phases (MGDG
428 and NAPE) in fermented dough to decrease significantly, thereby producing lipids promoting lamellar
429 (MGMG and NALPE) and hexagonal I (FFA) phases. This resulted in significant LV increase (Figure 1).
430 At 200 ppm lipase dosage, LV was maximal (Figure 1). Our results do not allow to pinpoint whether
431 the level of lipids promoting hexagonal II or cubic phases increased or decreased in fermented dough since

certain lipids promoting these mesophases were degraded (*e.g.* MGDG and NAPE) whereas others were produced (*e.g.* MAG) and lipid analyses could only be semi-quantitative. The same holds for the level of lipids promoting lamellar phases. We presume that due to extensive FFA production the level of lipids promoting hexagonal I phases increased. Thus, the improved LV is most likely caused by an improved balance between, on the one hand, lipids promoting hexagonal I phases and, on the other hand, non-polar lipids and lipids promoting hexagonal II or cubic phases. It may also be caused by an increased level of lipids favoring the lamellar phase.

At a dosage of 3,000 ppm lipase, the levels of lipids promoting hexagonal II or cubic and lamellar phases were clearly reduced whereas the level of lipids favoring hexagonal I phases (FFA) increased. Here, an insufficient level of lipids promoting lamellar phases and an imbalance between, on the one hand, lipids favoring hexagonal I phases and, on the other hand, non-polar lipids and lipids favoring hexagonal II or cubic phases resulted in an inferior LV (Figure 1).

The present results demonstrate that an appropriate balance between different types of lipids in terms of them promoting formation of different mesophases (in aqueous phases) and monolayers (at gas/liquid interfaces) is crucial for optimal stability of gas cells in bread dough. First, sufficient levels of lipids promoting lamellar phases which, in turn, yield stable condensed monolayers at gas cell interfaces, need to be present. Second, a suitable mixture between, on the one hand, lipids favoring hexagonal I phases and, on the other hand, non-polar lipids and lipids favoring hexagonal II or cubic phases is essential. This then allows lipids favoring hexagonal I phases to promote gas cell stabilization either by emulsifying (deleterious) non-polar lipids or by creating stable monolayers with lipids favoring hexagonal II or cubic phases as a result of their compatible shape.

We assume that in wheat flour BM levels of lipids promoting the hexagonal II or cubic phase are too high whereas those of lipids promoting the lamellar and hexagonal I phases are too low to result in optimal gas cells stability. Lipases cause a change in the balance between different types of lipids (*i.e.*

456 favoring different types of mesophases and monolayers) endogenously present. A schematic overview of
457 the mechanism which we put forward to describe the role of wheat endogenous lipids and their
458 enzymatically released hydrolysis products in BM is provided in Figure 5. As far as we know, this is the first
459 time that a schematic representation of the mechanism whereby wheat endogenous lipids and their
460 enzymatically released hydrolysis products impact bread making is presented.

461 Certain aspects of this mechanism have already been outlined by Gerits et al. (2014) and
462 Schaffarczyck et al. (2016) and were used to explain the positive impact of certain lipases on LV in bread
463 making with two flour samples and several lipases other than the one used in the present study. In
464 addition, we propose that lipase exerts a positive effect on the capability of lipids to adsorb at the
465 gas/liquid interface by introducing "defects" in liposomes or micelles (Figure 5). These defects are
466 presumably generated when (part of) the lipids building a structure (*e.g.* a liposome or micelle) are
467 enzymatically modified (*e.g.* during fermentation). This weakens the forces keeping the lipid structure
468 together and increases the rate of adsorption of surface-active lipids at the gas cell interfaces.

469

470 **5 Conclusions**

471 MGDG and NAPE were identified as the most accessible substrates of the lipase from *F. oxysporum*
472 used in BM in the present study. Hydrolysis of these most accessible substrates and thus release of
473 MGMG, NALPE and FFA was largely if not entirely responsible for LV increases when applying the lipase in
474 BM. Lipase overdosage caused endogenously present lipids as well as enzymatically released lysolipids to
475 be hydrolyzed and LV to decrease. An appropriate balance between endogenously present lipids, their
476 corresponding lysolipids and FFA is thus of major importance in BM.

477 Lipases cause a change in the balance between different types of lipids (*i.e.* favoring different types
478 of mesophases and monolayers) that are endogenously present. An optimal BM lipase preferably
479 hydrolyzes lipids promoting hexagonal II or cubic phases into lipids promoting lamellar phases (*e.g.*

hydrolysis of MGDG and NAPE into MGMG and NALPE). Its dosage should be such that the amount of lipids promoting the lamellar phase, yielding stable condensed monolayers, is maximal while maintaining an appropriate balance between lipids promoting hexagonal I phases, non-polar lipids and lipids promoting hexagonal II or cubic phases. Finally, lipases improve the capability of lipids to adsorb at gas/liquid interfaces by introducing "defects" in liposomes or micelles.

The mechanism whereby wheat endogenous lipids and their enzymatically released hydrolysis products impact BM proposed here provides a plausible theoretical explanation for the positive impact of certain lipases on LV. Further research can validate this mechanism in larger scale BM with other flour samples and/or other successful BM lipases. Furthermore, experimental evidence proving this theoretical mechanism needs to be supplied. The dough liquid phase, also referred to as dough liquor, can be obtained by ultracentrifugation of dough and is assumed to contain the liquid films surrounding gas cells with their dispersed surface active components (Baker, Parker, & Mize, 1946; Gerits, et al., 2015). Therefore, analyses of dough liquor lipid composition, surface tension, surface viscoelasticity and types of mesophases and/or monolayers formed should allow validating the mechanism whereby wheat endogenous lipids and their enzymatically released hydrolysis products act in BM.

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Conflict-of-interest statement

The authors declare no conflict-of-interest.

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Figure captions

Figure 1: Change in specific loaf volume (LV) as a function of lipase level for bread prepared with lipase from *Fusarium oxysporum*. Changes in specific LV were calculated as the difference between the specific LV of bread prepared with and without lipase divided by the specific LV of bread prepared without lipase (3.1 cm^3/g ± 0.1 cm^3/g) and expressed as percentages. Lipase levels are expressed in ppm as explained in §2.3. Average values with corresponding ranges of duplicate measurements are shown.

Figure 2: Free lipid levels, expressed as relative peak areas in arbitrary units (A.U.), in freshly mixed (white bars) and in fully fermented (gray bars) dough prepared with and without different levels of lipase from *Fusarium oxysporum*. Lipase levels are expressed in ppm as explained in §2.3. Averages with corresponding standard deviations of triplicate measurements are shown. For each lipid class and per type of dough sample (freshly mixed, fully fermented), bars with differing capital letters are significantly different from each other ($p < 0.05$). For each lipid class and per lipase level (0, 20, 200, 3,000 ppm), bars with differing small letters are significantly different from each other ($p < 0.05$). TAG, triacylglycerols; FFA, free fatty acids.

Figure 3: Bound lipid levels, expressed as relative peak areas in arbitrary units (A.U.), in freshly mixed (white bars) and in fully fermented (gray bars) dough prepared with and without different levels of lipase from *Fusarium oxysporum*. Lipase levels are expressed in ppm as explained in §2.3. Averages with corresponding standard deviations of triplicate measurements are shown. For each lipid class and per type of dough sample (freshly mixed, fully fermented), bars with differing capital letters are significantly different from each other ($p < 0.05$). For each lipid class and per lipase level (0, 20, 200, 3,000 ppm), bars with differing small letters are significantly different from each other ($p < 0.05$). TAG, triacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; MGDG, monogalactosyldiacylglycerols; MGMG, monogalactosylmonoacylglycerols; DGDG, digalactosyldiacylglycerols; DGMG, digalactosylmonoacylglycerols; NAPE, N-acyl phosphatidylethanolamines; NALPE, N-acyl

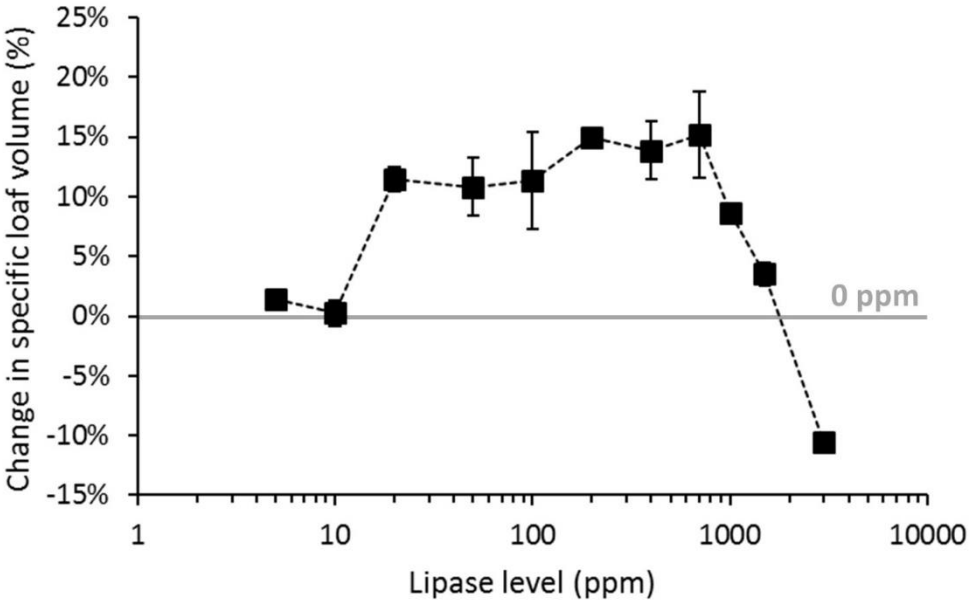
618 lysophosphatidylethanolamines; PC, phosphatidylcholines; LPC, lysophosphatidylcholines; nd, not
619 detected.

620 **Figure 4:** An overview of the possible orientations of lipids at gas/liquid interfaces (A) and in
621 aqueous phases (B) in wheat flour bread making [based on Sroan et al. (2009), Selmair (2010), Gerits et
622 al. (2014) and Schaffarczyk et al. (2016)]. The dotted lines indicate the most favorable orientations with
623 regard to gas cell stability in bread making. HLB, hydrophilic-lipophilic balance.

624 **Figure 5:** Schematic representation of fermented wheat flour dough without (A) and with (B) lipase
625 from *Fusarium oxysporum* applied at optimal level, illustrating the theoretical mechanism whereby wheat
626 endogenous lipids and their enzymatically released hydrolysis products exert their impact in bread
627 making. (A) In bread making without lipases, levels of lipids promoting hexagonal II or cubic phases are
628 too high whereas those of lipids promoting lamellar and hexagonal I phases are too low to result in optimal
629 gas cell stabilization. (B) The lipase hydrolyzes lipids promoting hexagonal II or cubic phases into lipids
630 promoting lamellar and hexagonal I phases. Optimal gas cell stabilization is achieved when the level of
631 lipids promoting lamellar phases, yielding stable condensed monolayers, is maximal while maintaining an
632 appropriate balance between, on the one hand, lipids promoting hexagonal I phases and, on the other
633 hand, non-polar lipids and lipids promoting hexagonal II or cubic phases. Finally, lipases promote
634 adsorption of lipids at gas/liquid interfaces by introducing defects in existing micelles and liposomes.

635 **Figures**

636 Figure 1



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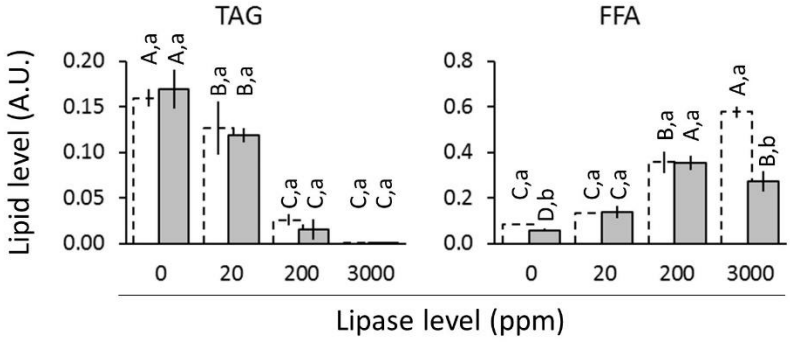
638 Figure 2



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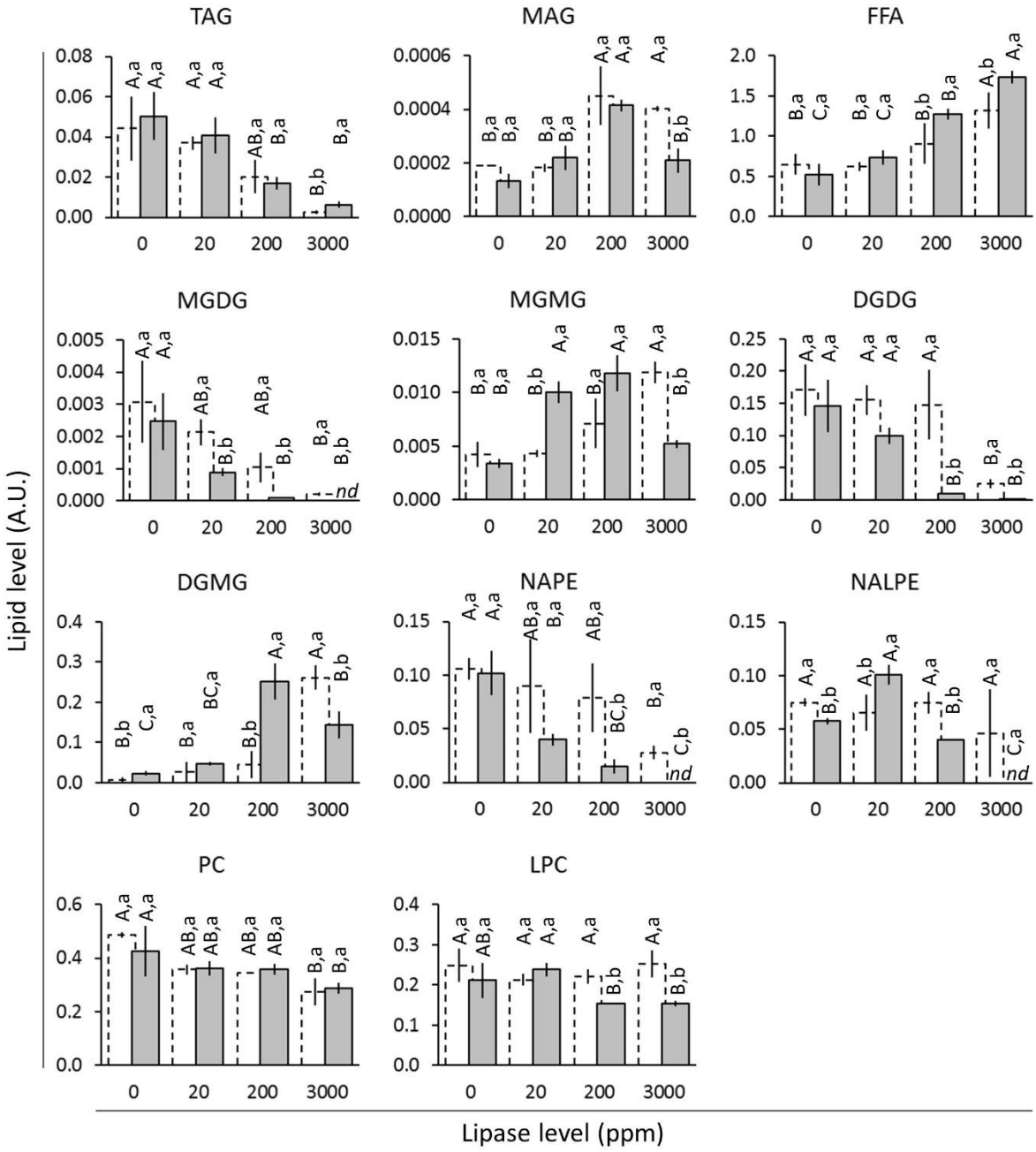
640 Figure 3



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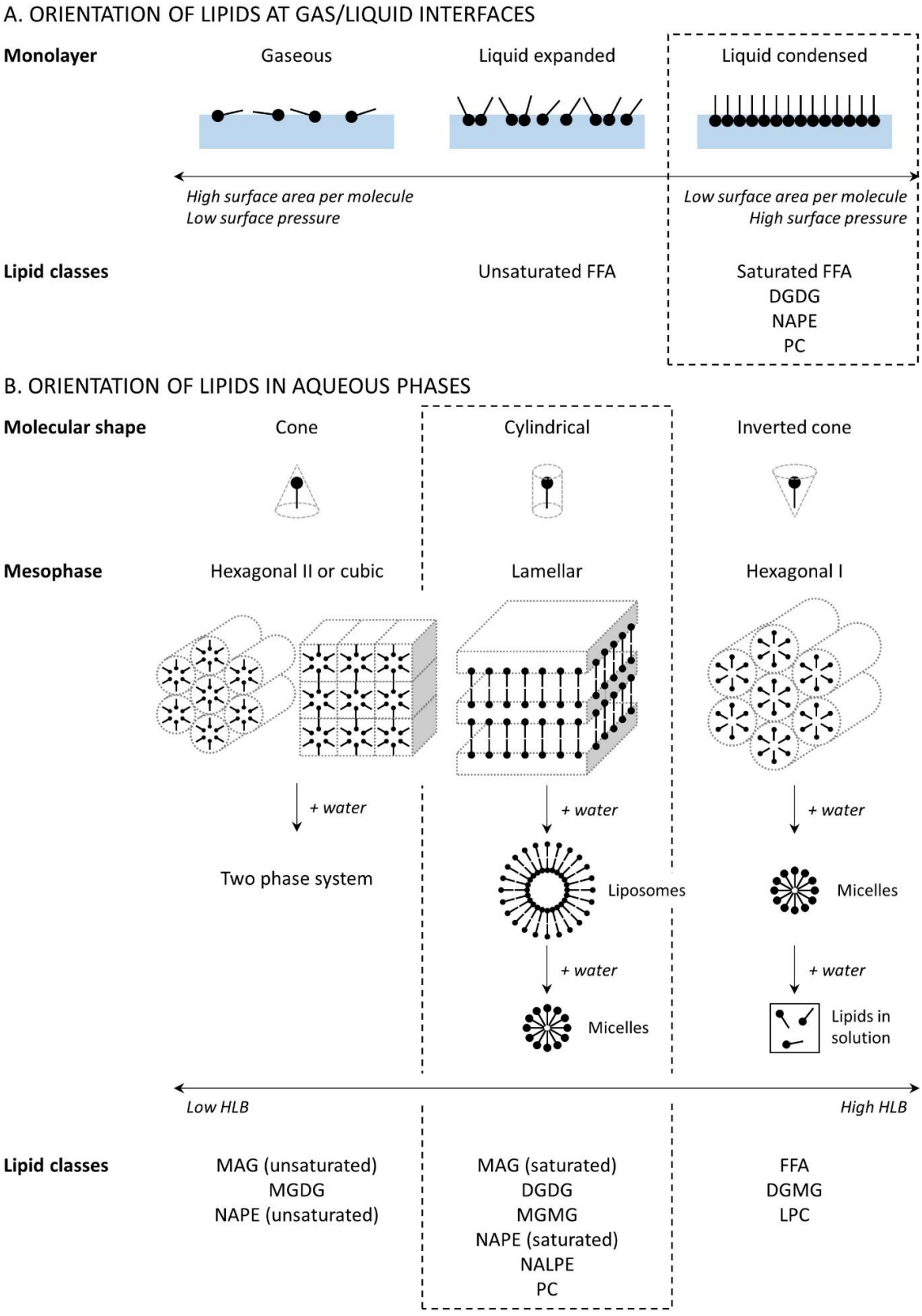
642 Figure 4



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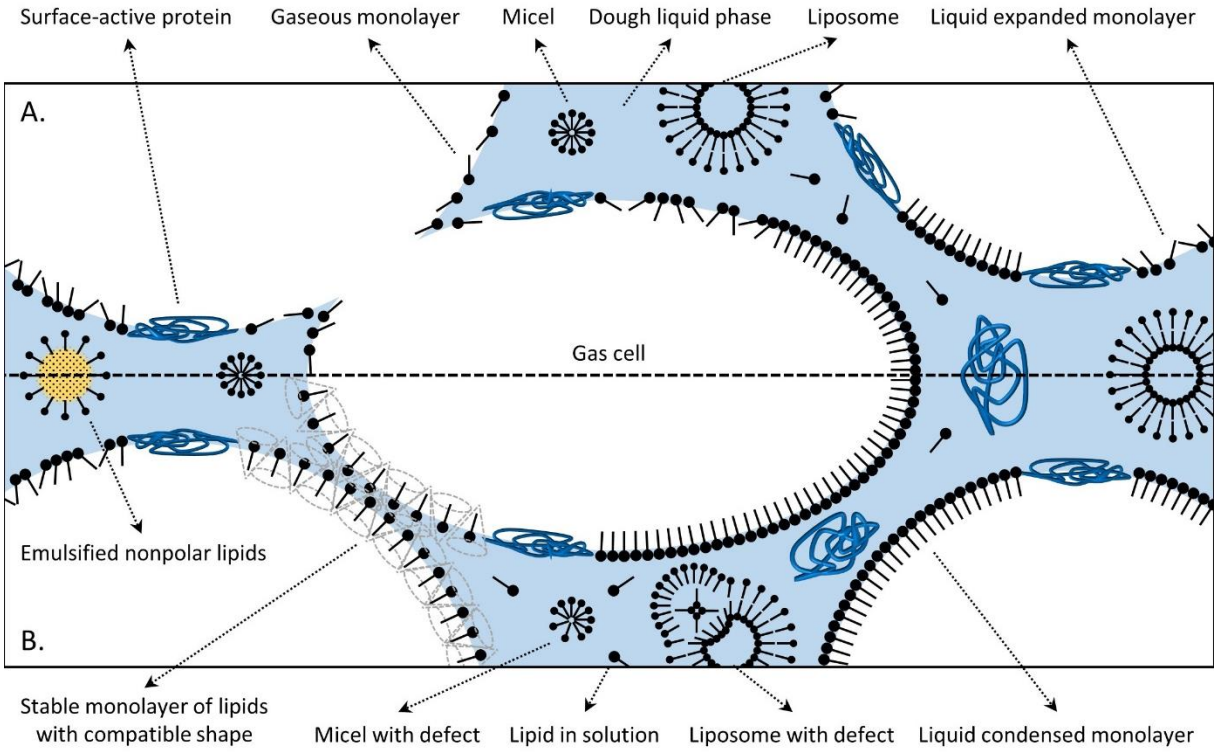
644 Figure 5



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