Thin film drainage dynamics of wheat and rye dough liquors and oat batter liquor

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Abstract

To obtain bread with a high loaf volume and homogeneous crumb, gas cells require sufficient stability until the discontinuous gas structure of dough is transformed into the continuous structure of bread. At the end of fermentation, dough gas cells are supposedly regionally separated by two air-water (A-W) interfaces and a liquid film. ‘Dough liquor’ (DL) or ‘batter liquor’ (BL), i.e. the supernatant after ultracentrifugation of dough and batter, respectively, is a model system for the fluid in these films. We report here on drainage dynamics of free-standing thin films (TFs) prepared from wheat and rye DLs and oat BL after applying a driving pressure of either 50 or 200 Pa at bulk concentrations lower than and equal to (‘native’) those in the respective supernatants obtained by ultracentrifugation. At lowered bulk concentrations, wheat and rye DL TFs were surrounded by planar and dimpled A-W interfaces, respectively, both of which were composed of weakly viscoelastic mixed protein-lipid films. In contrast, lipids stabilized dimpled oat BL TFs by migrating along their A-W interfaces and thus by generating Marangoni stresses. Liquid drainage and subsequent film rupture occurred rapidly in these TFs. At native bulk concentrations, TFs generally drained much slower and they were even found to be stable at small thicknesses (< 100 nm). Here, lipids stabilized dimpled wheat DL and oat BL TF A-W interfaces by Marangoni effects or by forming a densely-packed layer, respectively. In addition, wheat DL and oat BL TFs at their native concentrations exhibited stratification due to layered structuring of micelles, which contributed to TF stability by exerting oscillatory structural forces. Thus, it can here be concluded that wheat DL and oat BL constituents at their native concentrations may contribute substantially to stabilizing gas cells in dough.

Keywords

Dough liquor; batter liquor; proteins; lipids; thin film balance; interferometry

1. Introduction

Bread loaf volume and crumb structure depend on the total number and size distribution of gas cells incorporated during dough formation and on how they are stabilized during dough fermentation and baking. In wheat bread making, the viscoelastic gluten protein network provides gas cells with structural support (van Vliet, Janssen, Bloksma and Walstra, 1992). It has been proposed that this network locally ruptures as a result of its substantial extension upon dough expansion in the late stages of fermentation and thus fails to enclose some gas cell areas (Gan *et al.* 1990). However, the extent to which dough films in between gas cells are ruptured likely depends on the proofing time. Nonetheless, from the moment such rupture occurs gas cells are believed to be stabilized only by a liquid film containing bulk [i.e. amongst others, non-starch polysaccharides (NSPs)] and surface-active (i.e. proteins and lipids) constituents (Gan, Ellis and Schofield, 1995). Over the past two decades, further evidence has been brought forward to support this concept (Dubreil, Biswas and Marion, 2002; Li, Dobraszczyk and Wilde, 2004; Turbin-Orger *et al.* 2015a). It thus seems that at the end of fermentation at least some gas cells in dough are separated only by a liquid film enclosed by two air-water (A-W) interfaces.

The liquid film constituents are believed to be part of the aqueous phase of dough. At least a certain fraction of this phase can be isolated from wheat dough by ultracentrifugation (Baker, Parker and Mize, 1946; Mauritzen and Stewart, 1965; MacRitchie, 1976; Larsson and Eliasson, 1996). Noteworthy is that the total amount of DL recovered by ultracentrifugation strongly depends on the total dough water content (Larsson and Eliasson, 1996; Sahi, 2003) and even linearly increases above a certain wheat dough water absorption (Larsson and Eliasson, 1996). The obtained supernatant is generally referred to as 'dough liquor' (DL) and has long been used as a model system for investigating the properties of wheat dough aqueous phase constituents. Most research in this regard indeed relates to DL isolated from wheat dough (MacRitchie, 1976; Sahi, 1994; Mills, Wilde, Salt and Skeggs, 2003; Sahi, 2003; Primo-Martín, Hamer and de Jongh, 2006; Salt *et al.* 2006; Pauly, Pareyt, Fierens and

Delcour, 2014; Turbin-Orger *et al.* 2015b; Salt *et al.* 2018; Melis, 2019). Today's consumers are interested in consuming a variety of bread products. Examples of such product types are non-wheat breads. However, non-wheat breads often have poorer loaf volume and crumb structure than wheat bread because they lack the typical wheat gluten network in their doughs or batters. Hence, it can be reasoned that the presumed mechanism of gas cell stabilization by dough or batter aqueous phase constituents may be even of greater importance in non-wheat than in wheat bread making. In recent work from our group, the composition and A-W interfacial characteristics of wheat, rye and oat DLs were investigated in-depth (Janssen, Wouters, Pauly and Delcour, 2018; Janssen *et al.* 2020a; Janssen *et al.* 2020c). Briefly, it was concluded that wheat DL stabilized A-W interfaces are composed of a weakly viscoelastic mixed protein-lipid film. Moreover, it was found that arabinoxylan (AX), the most abundant NSP in wheat flour, weakens the adsorbed wheat DL film by interacting with its constituents (Janssen *et al.* 2020a). Rye DL constituents form a strongly viscoelastic protein-dominated interfacial film (although some lipids were also present), which is further strengthened in the presence of AX. At oat DL stabilized A-W interfaces, a lipid-dominated film not affected by β -D-glucan, the major NSP in oat flour, is present (Janssen *et al.* 2020a). Despite this in-depth characterization of the A-W interfacial properties of these DLs, it remains difficult to directly relate these findings to bread loaf volume or crumb characteristics. It should be mentioned that in these studies (Janssen *et al.* 2018; Janssen *et al.* 2020a; Janssen *et al.* 2020c) as well as in previous reports in literature (MacRitchie, 1976; Sahi, 1994; Mills *et al.* 2003; Sahi, 2003; Primo-Martín *et al.* 2006; Salt *et al.* 2006; Pauly *et al.* 2014; Turbin-Orger *et al.* 2015b; Salt *et al.* 2018; Melis, 2019), A-W interfacial measurements were conducted using DLs at significantly lower concentrations than those occurring in the supernatant after ultracentrifugation. This mostly relates to the fact that the accuracy and sensitivity of the used interfacial dilatational and shear rheological measurements considerably decrease at higher sample bulk phase viscosities (η_b).

Moreover, whether a foam is stable or not does not merely depend on the composition of the A-W interfaces surrounding the gas cells but also on the rate at which liquid drains from the thin films (TFs) between these A-W interfaces. In food science, the focus of free-standing TF drainage studies

has been on isolated proteins such as casein (Marinova *et al.* 1997; Dimitrova, Leal-Calderon, Gurkov and Campbell, 2001; Cascão Pereira, Johansson, Radke and Blanch, 2003; Marinova *et al.* 2014; Chen *et al.* 2017; Chen *et al.* 2018; Obeid *et al.* 2019), bovine serum albumin (Marinova *et al.* 1997; Dimitrova *et al.* 2001; Cascão Pereira *et al.* 2003; Marinova *et al.* 2014; Lin *et al.* 2018), β -lactoglobulin (Dimitrova *et al.* 2001; Basheva, Gurkov, Christov and Campbell, 2006; Rullier, Axelos, Langevin and Novales, 2009, 2010; Gochev, Retzlaff, Exerowa and Miller, 2014; Gochev *et al.* 2020), and whey proteins (Marinova *et al.* 2009). TFs separated by mixed protein-surfactant stabilized A-W interfaces have also been studied (Wilde, Rodriguez niño, Clark and Rodriguez Patino, 1997; Husband and Wilde 1998; Sarker, Wilde and Clark, 1998; Maldonado-Valderrama and Langevin, 2008). Last but not least, Senée, Robillard and Vignes-Adler (1999) have studied the drainage of TFs from Champagnes.

In most of the above studies, the focus has been on TFs at equilibrium. This means that the TF is slowly drained until the disjoining pressure (Π) equals the capillary hydrostatic pressure (P_c) (Bergeron and Radke, 1992). In this manner, the behavior at equilibrium is studied. However, this approach does not allow assessing the dynamics of the TF drainage process and the role of hydrodynamic forces in particular. However, these dynamics come into play when gas cells approach each other and even to a large extent determine when TF rupture and, subsequently, coalescence occur (Dagastine *et al.* 2006; Vakarelski *et al.* 2010; Chatzigiannakis, Veenstra, ten Bosch and Vermant, 2020).

At large TF thickness (> 100 nm), the disjoining pressure (Π) is negligible and the rate at which liquid drains from a TF between two A-W interfaces under an applied P_c depends on the:

- 1. capillarity and hydrodynamics (Fig 1A).** Applying a P_c may deform the A-W interfaces of a TF into a so-called ‘dimple’. This implies a concave shape of the A-W interfaces surrounding the TF when looked at from within. Dimpled A-W interfaces are the result of an increased hydrodynamic pressure (P_H) in the center of the TF (Frankel and Mysels, 1962; Ivanov and Kralchevsky, 1997). The pronounced curvature of a dimpled TF decreases P_c . This results in a reduced rate of liquid drainage as compared to that from between two planar interfaces (Joye, Hirasaki and Miller, 1992). As liquid drainage proceeds in such system, the dimple may be

‘washed out’ from the TF, leading to two planar A-W interfaces (although small thickness corrugations are often observed) (Joye, Hirasaki and Miller, 1994; Kannan, Shieh, Leiske and Fuller, 2018). This washing out process is believed to be a surface tension (σ) driven hydrodynamic instability which accelerates liquid drainage and is therefore considered detrimental for TF stability (Joye *et al.* 1994).

2. stress-boundary conditions (Fig 1B). The rate at which liquid drains from a system depends on the gradient of the velocity field at the natural boundaries of the flow domain, in this case the A-W interfaces surrounding the TF (Fuller and Vermant, 2012). In most instances, liquid drains slower if these A-W interfaces possess more rheological stress as this would imply that they are more resistant to deformations (Hermans, Bhamla Saad, Kao, Fuller and Vermant, 2015). **Proteins** when adsorbed do so by developing a strongly viscoelastic film thereat (Murray, 2007). In contrast, **lipids** form a weakly viscoelastic monolayer at the A-W interface. When their interfacial concentration is locally reduced by surface convection, e.g. when the A-W interfaces surrounding the TF are deformed into a dimple (see above and **Fig 1A**), lipids are inclined to restore this spatial concentration and thus σ gradient. As such, they generate counteracting Marangoni stresses which decrease the rate of surface convection (Manikantan and Squires, 2020). Lipid molecules insoluble in the bulk phase do so by diffusing to regions of lower surface concentration and thus higher σ . Lipids soluble in the bulk phase can generate Marangoni stresses which depend on the rate of adsorption/desorption of these lipids at the A-W interface (Manikantan and Squires, 2020). Mixed protein-lipid interfaces generally have less rheological stress than A-W interfaces stabilized by proteins or lipids alone as proteins hinder lipids from generating Marangoni stresses while lipids prevent the formation of a coherent viscoelastic protein layer (Wilde, 2000).

Fig 1. Graphical illustration of the interfacial and bulk phenomena determining the rate at which liquid drains from a thin film (TF) under a capillary hydrostatic pressure (P_c). At TF thickness values exceeding 100 nm, the rate of liquid drainage largely depends on the shape (A) and the nature of the stress-boundary conditions (stress-free or stress-carrying) (B) of the air-water (A-W) interfaces. At lower TF thickness values, the magnitude of the disjoining pressure (Π) influences drainage rates and final stability. Π depends on the contributions of (C1) supramolecular interactions (Π_{sup}), and the associated oscillatory structural force, and (C2) intermolecular interactions, which either stabilize [i.e. electrostatic (Π_{el}) or steric (Π_{st}) repulsive forces] or destabilize [i.e. van der Waals attractive forces (Π_{VDW})] the TF.

3. **magnitude of P_c (Eq 1)**, which is influenced by the pressure in the surrounding gas phase (P_g) and the Laplace pressure due to the curvature in the Plateau border region of the TF. In turn, both P_g and this Laplace pressure contribute to P_H (Valkovska, Danov and Ivanov, 2002).

4. **η_b** , according to the Stefan-Reynolds equation (1874; 1886):

$$\frac{dh}{dt} = \frac{-2h^3}{3\eta_b R_{TF}^2} (P_c - \Pi) \text{ (Eq 1)}$$

where t is the drainage time, h the TF thickness at time t , and R_{TF} the TF radius. TFs prepared from high η_b solutions drain slower than TFs prepared from low η_b DL solutions. For DL TFs, η_b is mostly determined by the concentration and structure of NSPs (Janssen *et al.* 2020a). **Eq 1** is only valid for planar films of constant radius, and for the case of a no slip boundary condition. Although more elaborate models are required when the films become deformed (Dagastine *et al.* 2006; Vakarelski *et al.* 2010), the Stefan-Reynolds equation identifies the main factors related to film hydrodynamics, i.e. the thickness decrease is driven by the pressure drop and slowed down by the bulk viscosity.

At small TF thickness (< 100 nm), the rate of liquid drainage strongly depends on the magnitude of Π . In turn, the contribution of Π is function of the TF thickness and originates from supramolecular (Π_{sup}) (**Fig C1**) (Nikolov and Wasan, 1989; Bergeron, Jimenez-Laguna and Radke, 1992; Bergeron and Radke, 1992) and intermolecular (**Fig C2**) (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) interactions between adsorbed constituents at both A-W interfaces. The former come into play when the TF is made up of one or more layers of supramolecular structures such as micelles (Nikolov and Wasan, 1989; Bergeron *et al.* 1992), or (synthetic) nanoparticles (Sethumadhavan, Nikolov and Wasan, 2001). The latter include attractive Van der Waals forces (Π_{VDW}), repulsive electrostatic forces (Π_{el}), and repulsive steric forces (Π_{st}) (Damodaran, 2005).

To fully understand gas cell stabilization in bread dough, insight in the drainage of liquid films between such gas cells would be of great interest. Against this background, for the first time, the drainage of wheat, rye, and oat DL TFs were assessed by interferometry using a bike-wheel shaped microcell similar to that of Cascão Pereira, Johansson, Blanch and Radke (2001), with modifications

enabling assessing drainage dynamics over a wide range of driving pressures (Chatzigiannakis *et al.* 2020). TF measurements were performed with DLs (i) at bulk concentrations at which they occur in the respective supernatants after ultracentrifugation ('native concentration') and (ii) at lowered bulk concentrations. While the former measurements are the more relevant ones in the context of bread making, the latter allow evaluating the extent to which the outcome aligns with literature reports dealing with A-W interfacial properties of diluted DL samples.

2. Materials & Methods

2.1 Materials

Crousti bread wheat flour was purchased from Dossche Mills (Deinze, Belgium). Rye kernels (cultivar Dukato) from AVEVE (Merksem, Belgium) were conditioned to 16.0% moisture and subsequently roller milled in-house using a Bühler (Uzwil, Switzerland) MLU-202 laboratory mill as in Delcour, Vanhamel and De Geest (1989). Commercial oat flour was from Raisio Nutrition (Raisio, Finland).

Sugar, salt, and fresh compressed yeast (AB Mauri, Dordrecht, Nederland) were bought in a local supermarket. All chemicals, solvents, and reagents were at least of analytical grade.

A xylanase preparation (from *Aspergillus aculeatus*, Shearzyme 500L, containing 30,000 xylanase units/mL) was from Novozymes (Bagsvaerd, Denmark). Xylanase from *A. aculeatus* preferentially hydrolyzes the water-extractable AX population of wheat flour (Courtin and Delcour, 2001). A similar substrate specificity has been observed with rye flour water-extractable AX (unpublished results). Lichenase (from *Bacillus subtilis*, containing 1,000 lichenase units/mL) and β -D-Glucosidase (from *Aspergillus niger*, containing 40 β -D-glucosidase units/mL) preparations were from the " β -D-glucan assay" kit of Megazyme (Bray, Ireland). Lichenases convert β -D-glucan into β -D-glucoligosaccharides by catalyzing the hydrolysis of β -1,4 bonds. β -D-glucosidases then selectively degrade

these β -D-glucosaccharides into D-glucose molecules by catalyzing the hydrolysis of both β -1,3 and β -1,4 bonds (McCleary and Nurthen, 1986).

2.2 Methods

2.2.1 Dough making and dough liquor isolation

Wheat dough optimum water level and mixing time were determined using AACCI Methods 54-21.02 and 54-40.02, respectively. The bread making procedure was essentially that described by Finney (1984). Wheat flour (100.0 g), deionized water (54.73 mL), sugar (6.0 g), yeast (5.3 g), and salt (1.5 g) were mixed in a pin mixer (National Manufacturing, Lincoln, NE, USA) during 210 s. In the case of rye and oat breads, the optimum water level was selected based on preliminary baking trials in which the water level resulting in the highest specific bread loaf volume was considered as optimal. In this case, the protocol in essence was that described by Hager, Bosmans and Delcour (2014). Rye or oat flour (265.0 g or 236.0 g, respectively), deionized water (200.9 mL or 232.3 mL, respectively), sugar (16.0 g or 14.8 g, respectively), yeast (14.1 g or 13.1 g, respectively), and salt (4.0 g or 3.7 g, respectively) were mixed in a KitchenAid (St. Joseph, MI, USA) KPM5 mixer (60 s at speed 1 and 120 s at speed 4). All doughs/batters were fermented in a National Manufacturing fermentation cabinet (30.0 °C, 90% relative humidity, 126 min). Wheat doughs were sheeted in a National Manufacturing sheeter thrice during fermentation (roll gaps 4.8 mm after 52 and 77 min and 7.9 mm after 90 min). Hydrating and mixing rye or oat flour results in poorly extensible dough and a cake-like batter, respectively. Therefore, rye doughs and oat batters could not be sheeted. While Janssen *et al.* (2018; 2020a;2020c) referred to the supernatant recovered from oat batter by ultracentrifugation as ‘oat DL’, we here more correctly refer to it as ‘oat batter liquor’ (oat BL). Fermented dough samples (ca. 15.0 g, accurately weighed) were centrifuged at 165,000 *g* (20 °C, 60 min) in 38 mL thick polycarbonate tubes (Beckman Coulter, Brea, CA, USA) with an L7 Ultracentrifuge (Beckman Coulter) and thereafter immediately placed on ice to minimize potential endogenous enzymatic activity. The supernatants

(the DLs/BL) were collected in tared amber-colored Falcon tubes, weighed, flash frozen with liquid nitrogen, freeze-dried, and stored at -18 °C until analysis. In previous work (Janssen, 2020b) it was established that freeze-drying does not alter the A-W interfacial properties of DL/BL constituents. These freeze-dried wheat and rye DL and oat BL powders were used for composition analysis (see section 2.2.2) and sample preparation (see section 2.2.3) prior to TF drainage measurements (see section 2.2.4).

2.2.2 Dough/batter liquor chemical composition

DL/BL protein (N x 6.25) content was determined with an adaptation of AOAC method 990.03 to an automated 1108 Elemental Analyser (Carlo Erba, Hindley Green, UK) for microgram scale samples. DL/BL lipid levels were determined gravimetrically as in Janssen *et al.* (2020a; 2020c), based on the extraction protocol of Bligh and Dyer (1959). AX levels were determined as in Janssen *et al.* (2020a; 2020c) with a gas chromatography procedure in which flour samples were sequentially subjected to acid hydrolysis, reduction of monosaccharides to alditols, and subsequent conversion to alditol peracetates (Englyst and Cummings, 1984). β -D-Glucan levels were colorimetrically measured using a Megazyme (Bray, Ireland) protocol, which is also available as AACCI Method 32.23-01 (McCleary and Nurthen, 1986) as in Janssen *et al.* (2020a; 2020c). Protein, lipid, AX, and β -D-glucan levels were determined in duplicate on DLs/BLs from each of two separate isolations.

2.2.3 Sample preparation

TF measurements (see section 2.2.4) were carried out on wheat and rye DLs at 1.50 and 0.50 mg protein/mL, respectively, and oat BL at 1.00 mg protein/mL, as well as on wheat DL and oat BL at their native concentrations (40.1 and 7.0 mg protein/mL, respectively). In all cases, the most abundant NSP population (AX in wheat and rye DLs and β -D-glucan in oat BL) was depolymerized by enzyme(s) prior to performing TF measurements. Enzymatic treatments of wheat and rye DLs and oat BL were as follows. Freeze-dried wheat and rye DL powders were dissolved in deionized water (continuous

stirring, 30 min, 23 ± 2 °C) at concentrations of 1.50 and 0.50 mg protein/mL, respectively. Xylanase solution [containing 1.0 mL of xylanase preparation (see section 2.1) in 100 mL water] was added under gentle stirring to obtain a ratio of xylanase solution/DL dm protein of 2/1 (v/w). Freeze-dried oat BL powder was dissolved in deionized water (continuous stirring, 30 min, 23 ± 2 °C) at a concentration of 1.00 mg protein/mL. Then, a solution of both lichenase and β -D-glucosidase [containing 0.50 mL of both the lichenase and β -D-glucosidase preparations (see section 2.1) in 100 mL water] was added under gentle stirring to obtain ratios of enzyme solution/DL dm protein of 1/1 (v/w) for each of the enzymes present. The respective enzymatic treatment of wheat DL and oat BL at their native concentrations was as above with the exception that the ratio of total enzyme solution/DL or BL dm protein was 10/1 (v/w) instead of 2/1 (v/w). The sample volume required for TF measurements was approximately 5.0 mL.

2.2.4 Analysis of dough/batter liquor thin film drainage dynamics

TF measurements were carried out on the wheat and rye DL and oat BL samples described in section 2.2.3. **Fig 2** shows the TF balance set-up used. The design of the bike-wheel shaped microchip was based on that of Cascão Perreira *et al.* (2001) and identical to that of Beltramo and Vermant (2016). More recently, Chatzigiannakis *et al.* (2020) modified the TF set-up allowing assessing drainage dynamics by reducing compliance of the setup and adding adequate pressure control systems. It is this modified TF set-up that was used in the present study. The microchips were made on borosilicate glass by photolithography (Micronit Microfluidics, Enschede, The Netherlands) and consisted of a diamond bit drilled central orifice (diameter: 1 mm, thickness: 400 μ m) connected to a larger circular channel (width: 500 μ m, depth: 50 μ m) via 25 smaller channels (width: 45 μ m, depth: 20 μ m, length: 800 μ m) oriented in a radial pattern. The chip was glued to the top of a titanium T-shaped holder using two component epoxy adhesives (**Fig 2A**). Before use, the walls of the bike-wheel chip were rendered hydrophilic by immersion and ultra-sonication in sodium hydroxide saturated ethanol. After creating a DL/BL thick film in the central orifice by means of a 1.0 mL syringe coupled to rigid

polytetrafluoroethylene tubing, the bike-wheel was placed into a pressure chamber (Fig 2B). Evaporation of the DL/BL sample was minimized by adding excess water to the chamber. The chamber was then thoroughly sealed to avoid leaks and connected to a piezoelectric pressure control system (Elveflow MK3+, Elvsys, Paris, France) via rigid polytetrafluoroethylene tubing (Fig 2C). The pressure control system has a resolution of 1 Pa and a response time of 10 ms.

Fig 2. Thin film (TF) interferometry set-up. **A.** Photograph of the hydrophilic bike-wheel glued to the T-shaped holder (Chatzigiannakis et al. 2020). **B.** Aluminum pressure chamber in which the bike-wheel is mounted. This chamber is connected to a piezoelectric pressure control system. **C.** Schematic side view of a dough liquor (DL) TF (in yellow) in the inner orifice of the bike-wheel. The end of the T-shaped holder to which the bike-wheel is glued is thoroughly sealed. **D.** Top view of the inner orifice of the bike-wheel. The radially oriented channels allow uniform drainage from the central orifice.

TF experiments were executed as follows. First, the TF was carefully drained by increasing P_g in small discrete steps until the first interference fringes appeared and the film remained stable, i.e. the TF neither thickened nor thinned. At this point, the contribution of Π to the total pressure in the TF (the difference between P_c and Π) is assumed to be negligible when the TF had a thickness > 100

nm. Thus, the first interference fringes remained stable because P_c was zero (Bergeron and Radke, 1992):

$$P_c = P_g + P_{L,bw} = 0 \text{ (Eq 2)}$$

$$P_c = P_g + \frac{2\sigma}{R_{bw}} = 0 \text{ (Eq 3)}$$

where $P_{L,bw}$ is the Laplace pressure due to the natural curvature of the film in the bike-wheel and where R_{bw} is the radius of the bike-wheel's hole (500 μm). Subsequently, drainage of the TF was forced by applying an excess P_g of either 50 Pa ($eP_g^{50\text{Pa}}$) or 200 Pa ($eP_g^{200\text{Pa}}$). From this point onwards, the TF reduced its thicknesses and there was a contribution of Π to the total pressure in the TF (the difference between P_c and Π , **Eq 1**). In addition, there was a contribution of hydrodynamic pressures (P_H) to P_c because liquid flow in the TF took place. Thus, liquid drains from the TF when:

$$P_c > \Pi \text{ (Eq 4), and thus when}$$

$$P_g + eP_g + \frac{2\sigma}{R_{bw}} - P_H > \Pi \text{ (Eq 5)}$$

From a certain TF thickness, the radius of the TF increases until it either ruptures or over time reaches an equilibrium radius ($R_{TF,eq}$). The latter is defined by the pressure balance at the Plateau border region of the TF (Toshev and Ivanov, 1975):

$$R_{TF,eq}^2 = R_{bw}^2 - \left(\frac{2\sigma R_{bw}}{eP_g + P_{L,bw}} \right) \text{ (Eq 6)}$$

Thus, in case no rupture takes place, a TF subjected to a larger eP_g expands to a larger $R_{TF,eq}$ than when a lower eP_g is applied.

In case no TF rupture took place in the time frame of the experiment, the bike-wheel was thoroughly rinsed with water and filled with new DL/BL material prior to initiating a new measurement. When it did rupture, a new thick film was formed by lowering the eP_g to 0 Pa and the measurement was repeated as outlined above. The latter was possible because the end of the holder to which the bike-wheel chip was glued was thoroughly sealed (**Fig 2C**). The total time required for preparing the sample and reaching the point at which P_c was zero (**Eq 3**) was approximately 8 min for all DL/BL samples. Thus, A-W interface aging was limited before drainage was forcibly induced. All

repetitions for a given DL/BL sample were executed within 30 min to prevent aging of the bulk phase. **Table S1 (supplementary information)** provides an overview of the total number of drainage experiments performed for the different samples.

Throughout the experiments, TFs were viewed with an upright microscope (Nikon Eclipse FN1, Nikon Instruments, Melville, USA) equipped with a 10x long working distance objective (numerical aperture of 0.30) and a camera (ORCA-flash4.0 CMOS, Hamamatsu Photonics, Herrsching, Germany) (**Fig 2D**). Images were stored in .tiff format (2048 x 2048 pixels) with a maximal temporal resolution of 10 ms and further processed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

A **drainage time** could only be determined in case of TF rupture and was defined as the time between the onset of TF expansion and rupture. When the TF ruptured, TF **expansion rates** were calculated by subtracting the initial TF radius (about 25 μm) from the TF radius just before rupture and dividing the resulting value by the drainage time. When the TF did not rupture, TF expansion rates were calculated by subtracting the initial TF radius from $R_{TF,eq}$ and dividing the resulting value by the time needed to reach $R_{TF,eq}$. A TF is expected to expand at a slower rate when the surrounding A-W interfaces provides more resistance to an applied deformation. To better understand this relationship, **Boussinesq (Bq) numbers** were calculated as:

$$Bq = \frac{|\eta_s^*|}{\eta_b R_{TF,av}} \text{ (Eq 7)}$$

where $|\eta_s^*|$ is the magnitude of the complex interfacial shear viscosity, taken here at a single frequency of 0.5 Hz and 8 min after the start of adsorption, and $R_{TF,av}$ the average TF radius of the drainage experiment. The η_s^* and η_b were determined with a standard rheometer equipped with a double wall ring (Vandebriel, Franck, Fuller, Moldenaers and Vermant, 2010) and double wall Couette geometry, respectively, as in Janssen *et al.* (2020a). For oat BL TFs, $|\eta_s^*|$ was lower than the detection limit of the set-up ($5.0 \times 10^{-6} \text{ Pa.s.m}$), so this value was used for calculating Bq numbers as an upper limit.

TF **equivalent thickness** calculations on the obtained images were based on interferometry using the Sheludko (1967) equation:

$$h_{eq} = \left(\frac{\lambda}{2\pi n_f} \right) \left(m\pi \pm \arcsin \sqrt{\frac{\Delta}{1+4Q \frac{(1-\Delta)}{(1-Q)^2}}} \right) \text{ (Eq 8)}$$

$$\Delta = \frac{I_f - I_{min}}{I_{max} - I_{min}} \text{ (Eq 9)} \quad Q = \left(\frac{n_f - n_c}{n_f + n_c} \right)^2 \text{ (Eq 10)}$$

with λ the wavelength of the monochromatic light used for reflection (508 nm), n_f and n_c the refractive indices of the TF (1.33, the refractive index of water) and air (1.00028), respectively, m the order of interference, I_f the intensity or grey value of one pixel of a particular TF image, I_{max} and I_{min} the maximum and minimum intensities or grey values, respectively, of the pixels of the TF region of all images obtained for a given TF drainage experiment (Exerowa and Kruglyakov, 1997). As the refractive indices were not determined, h_{eq} represents a calculated equivalent TF thickness of the pixel area analyzed. The TF h_{eq} values reported throughout this manuscript represent average values of several such areas of at least three different TF images (**Table S1**). It should be noted that TF thicknesses obtained via the Sheludko equation must be interpreted with care as it assumes (i) a planar film, (ii) constant I_{max} and I_{min} during the drainage experiment, (iii) constant refractive TF index, (iv) no adsorption, and (v) no secondary reflection (Sheludko, 1967).

3. Results and discussion

3.1 Dough liquor chemical composition

In previous work by our group, the chemical composition of the wheat and rye DLs and oat BL used here has been discussed in-depth (Janssen *et al.* 2018; 2020a; 2020b; 2020c). In short, lipid levels were high in oat BL (29.7% of BL dm) and much lower in rye (1.5% of DL dm) and wheat (0.5% of DL dm) DLs. The protein levels decreased in the order wheat (16.9% of DL dm), rye (14.0% of DL dm), and oat (7.8% of BL dm) DLs. AX was the major NSP in wheat and rye DLs (9.0 and 15.1% of DL dm,

respectively), while β -D-glucan was the most abundant NSP in oat BL (11.9% of BL dm). The aqueous phase of cereal doughs apart from proteins, lipids, and NSPs, contains mono-, di- and oligosaccharides (Primo-Martín *et al.* 2006; Salt *et al.* 2006) and probably also minor levels of minerals and vitamins (Belitz, Grosch and Schieberle, 2009).

3.2 Dough/batter liquor thin film drainage dynamics

TF drainage measurements were conducted both after applying eP_g^{50Pa} and eP_g^{200Pa} , which allowed mimicking the drainage of TFs separated by gas cells of different sizes. As a point of reference, the average radius of gas cells in dough increases from about 100 μ m up to 1000 μ m over the course of fermentation (Babin *et al.* 2006; Turbin-Orger *et al.* 2015a). Thus, according to the Young-Laplace equation:

$$P_{L, gas\ cell} = \sigma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \text{ (Eq 11)}$$

where $P_{L, gas\ cell}$ is the pressure difference across the gas cell A-W interface and R_1 and R_2 the gas cell radii in the x and y directions, respectively, perfectly spherical gas cells (for which R_1 equals R_2) with an average σ of 40 mN/m experience $P_{L, gas\ cell}$ values between 800 and 80 Pa during fermentation. As the gluten network surrounding gas cells presumably only ruptures during the late stages of fermentation, it seems reasonable to assume that gas cells regionally surrounded by a TF only indeed experience $P_{L, gas\ cell}$ values in the range of those applied in the present experiments. As the phenomena observed after applying eP_g^{50Pa} and eP_g^{200Pa} were very similar, the results obtained for eP_g^{200Pa} are discussed in-depth, while interferometry images obtained at eP_g^{50Pa} are provided as supplementary data.

First, the drainage dynamics of wheat and rye DL TFs at 1.50 and 0.50 mg protein/mL, respectively, and oat BL TFs at 1.00 mg protein/mL were analyzed. Although these concentrations were well below those in the respective supernatants after ultracentrifugation, these measurements allow rationalizing the findings reported in literature thus far (see section 1). Second, the drainage of wheat DL and oat BL TFs at their native concentrations was monitored to assess the potential of

aqueous phase constituents as they occur in dough to stabilize gas cells during bread making. Videos illustrating the drainage processes of all above-mentioned TFs, both after applying eP_g^{50Pa} and eP_g^{200Pa} , are included as **supplementary information**. Note that in case of the videos of TFs at native concentration one frame was selected every 9 s.

Some limitations of the experimental set-up used must be considered. First, rye DL TFs at their native concentration could not be monitored because rye DL constituents formed large aggregates in the microchip channels which prevented drainage to occur freely. Second, the TF balance set-up used was limited to liquids with sufficiently low η_b . Here, η_b was drastically reduced (Janssen *et al.* 2020a) by enzymatically depolymerizing the respective NSP populations (see section 2.2.3) prior to performing TF measurements. As mentioned earlier, η_b would directly affect TF drainage (**Eq 1**). In earlier work, η_b of the DLs/BL used in the present study have been reported on, which allows estimating their likely impact on TF drainage (Janssen *et al.* 2020a).

3.2.1 Drainage dynamics of wheat and rye dough liquor and oat batter liquor thin films at lowered bulk concentrations

3.2.1.1 Thin film structural changes

WHEAT DOUGH LIQUOR

Fig 3 shows interferometry images of wheat DL TFs at 1.50 mg protein/mL at different time points after applying eP_g^{200Pa} (**A** to **D**). The 3D intensity plot of the image just before rupture in **Fig 3D** is shown in **Fig 3E**. Just before rupture, at eP_g^{200Pa} , wheat DL TFs at 1.50 mg protein/mL were characterized by white regions near the Plateau border, whereas their central part consisted of white spots dispersed in a dark grey region (**Fig 3D**). In these measurements, such white and dark grey regions generally represent the thickest and thinnest part of the TF, respectively. The 3D pixel intensity plot of the image in **Fig 3D** (**Fig 3E**) further illustrates this. This thickness assumption is only valid when comparing TF regions with h_{eq} values in the same order of interference. For instance, the h_{eq} values of

the black regions in **Figs 3B** and **3C** have a greater thickness than the white regions in **Fig 3C** or dark grey regions in **Fig 3D** because the order of interference is higher in the former. Just before rupture (**Fig 3D**), the white regions near the plateau border and the dark grey regions in the TF had average h_{eq} values of 76 ± 5 nm and 20 ± 1 nm, respectively. The white regions likely consist of a relatively thick film surrounded by two A-W interfaces. In contrast, it is reasonable to assume that h_{eq} of the dark grey region represents the combined thicknesses of both A-W interfaces, as one can expect that at this point just before rupture little if any liquid remains between them. It was demonstrated in earlier work that at similar lowered bulk concentrations, both proteins and lipids are present at wheat DL stabilized A-W interfaces (Janssen *et al.* 2020c). Using interferometry and ellipsometry, the thicknesses of β -casein and bovine serum albumin TFs just before rupture have been estimated to be 17 – 26 nm and 4 – 12 nm, respectively, depending on the pH and ionic strength of the bulk phase, the aging time of the A-W interface, and the magnitude of eP_g (Cascão Pereira *et al.* 2003). Furthermore, h_{eq} of an inverse lipid bilayer or common black film corresponds to the sum of (i) twice the characteristic length of the hydrocarbon chains of the participating lipid molecules and (ii) the thickness of the aqueous layer separating both lipid monolayers. Common black films typically have h_{eq} values in the range of 10 – 20 nm, although the exact value depends on the contributions of Π_{VDW} and Π_{el} (Zhang, Yilixiati, Pearsall and Sharma, 2016). Based on the above for pure protein and lipid films, it thus seems plausible that the dark grey region in **Fig 3D**, which was about 20 nm thick, represents two opposing mixed protein-lipid A-W interfacial films, as was suggested in earlier work to be the case based on interfacial rheology measurements (Janssen *et al.* 2020c). The white spots dispersed in the central grey region had h_{eq} values between 60 and 70 nm (**Fig 3D**) and appeared as discrete entities in the 3D pixel intensity plot (**Fig 3E**). Such entities have previously been observed in TFs prepared from isolated proteins and have been interpreted to represent protein aggregates (Maldonado-Valderrama and Langevin, 2008; Rullier *et al.* 2009; Rullier *et al.* 2010; Gochev *et al.* 2014; Chen *et al.* 2018).

Fig 3. Interferometry images of wheat dough liquor (DL) thin films (TFs) at 1.50 mg protein/mL at different time points after applying an excess pressure of 200 Pa (eP_g^{200Pa}). D represents the center part of the image just before TF rupture and is presented in E as a 3D intensity plot, in which lower TF pixel intensities correspond to a lower equivalent thickness (h_{eq}), except for the black regions in B and the structures consisting of multiple concentric rings in A-D.

Additionally, black spots and relatively large structures surrounded by multiple concentric rings were observed (**Figs 3A to 3D**). Each ring corresponds to one order of reflected light interference or – in this case – an h_{eq} equal to $\lambda/4n$ or 95 nm. Thus, the number of concentric rings provides a rough estimate of the thickness at the center of those spots. In **Fig 3D**, the h_{eq} values at the center of the concentric ring structures varied between 145 and 320 nm. Thus, these concentric ring structures represented the thickest parts of wheat DL TFs at 1.50 mg protein/mL. Rullier *et al.* (2009; 2010) monitored the drainage of TFs prepared from solutions containing β -lactoglobulin aggregates and observed such concentric ring structures. Similar observations for protein aggregates were also made in other studies (Cascão Pereira *et al.* 2003; Chen *et al.* 2017; Chen *et al.* 2018). All in all, the concentric ring structures in **Figs 3A to 3D** likely represent larger protein aggregates than those in the earlier mentioned white and black spots. Interestingly, protein aggregates at the A-W interface coalesced when liquid drainage progressed, presumably a result of compression due to film thinning. At eP_g^{50Pa} (see **supplementary information**), smaller aggregates and no concentric ring structures were observed. This is probably related to less pronounced protein unfolding and subsequent aggregation at lower hydrostatic pressures (Dumay, Kalichevsky and Cheftel, 1994; Messens, Van Camp and Huyghebaert, 1997).

RYE DOUGH LIQUOR

Fig 4 shows interferometry images of rye DL TFs at 0.50 mg protein/mL at different time points after applying eP_g^{200Pa} (**A to D**). It was not possible to create a 3D intensity plot for rye DL TFs at eP_g^{200Pa} because the A-W interfaces of these TFs were dimpled (**Fig 1A**) and would have resulted in distorted 3D intensity plots. In addition, smaller black spots and concentric ring structures were observed (**Figs 4A to 4D**), with estimated h_{eq} values at their center ranging between 100 and 300 nm. As discussed above for wheat DL TFs, these concentric ring structures in rye DL TFs at 0.50 mg protein/mL (**Figs 4A to 4D**) most likely represent protein aggregates. Similar to what was observed for wheat DL TFs, protein aggregates at the A-W interfaces surrounding rye DL TFs coalesced during the drainage process, implying flow induced aggregation as the TF thinned.

Approximately 3 s after applying eP_g^{200Pa} , a white planar region appeared near the Plateau border (**Figs 4C and 4D**), with an average h_{eq} value of 79 ± 3 nm. It has been shown that proteins are the dominant constituents at rye DL stabilized A-W interfaces (Janssen *et al.* 2020c). However, the average h_{eq} of said white regions (79 nm at eP_g^{200Pa}) was much higher than that of protein stabilized A-W interfacial films [4 to 26 nm (Cascão Pereira *et al.* 2003)]. This indicates that these white regions not only represent the combined thicknesses of the A-W interfaces themselves but that there is still a liquid film containing DL constituents present between them.

Fig 4. Interferometry images of rye dough liquor (DL) thin films (TFs) at 0.50 mg protein/mL at different time points after applying an excess gas pressure of 200 Pa (eP_g^{200Pa}). D represents the image just before TF rupture. The dark grey regions in the white rectangular boxes are the result of steric and/or electrostatic repulsive forces occurring between proteins at the two opposing air-water interfaces.

Furthermore, small dark grey noncircular regions emerged near the Plateau border (highlighted by white boxes in **Fig 4D**) and expanded over time. These regions had an h_{eq} of 20 ± 1 nm, thus representing the part of the TF with the lowest thickness. Very similar observations were made at eP_g^{50Pa} (see **supplementary information**). As these spots appeared just before TF rupture, it can be assumed that merging of the A-W interfaces occurred in these regions. The onset and expansion of noncircular thin regions as observed in **Fig 4D** has earlier been described by Cascão Pereira *et al.* (2003) for β -casein and bovine serum albumin TFs and by Gochev *et al.* (2014) for β -lactoglobulin TFs. Establishing Π -area isotherms revealed that the occurrence of such regions is the result of both electrostatic and steric repulsive forces between proteins adsorbed at the A-W interfaces surrounding the TF (Cascão Pereira *et al.* 2003; Gochev *et al.* 2014). That proteins dominate rye DL stabilized A-W interfaces is in line with previous findings (Janssen *et al.* 2020a; Janssen *et al.* 2020c).

OAT BATTER LIQUOR

Fig 5 shows interferometry images of oat BL TFs at 1.00 mg protein/mL at different time points after applying eP_g^{200Pa} (**A** to **D**). 3D intensity plots could be made from oat BL TF interferometry images neither at eP_g^{50Pa} (see **supplementary information**) nor at eP_g^{200Pa} because the TFs were pronouncedly dimpled. Indeed, at eP_g^{200Pa} a symmetric dimple was observed in oat BL TFs at 1.00 mg protein/mL (**Figs 5A** and **5B**). It was visually observed that black and white spots migrated to the center of the dimple (**Fig 5A**). This indicates that the oat BL constituents in these spots were inclined to restore the σ gradient which originated from dimple formation (see section 1). This implies that Marangoni effects, which are typically observed for low molecular weight surfactant-stabilized interfaces (Damodaran, 2005), were at play here. Lipids thus likely dominated the A-W interfaces in oat BL TFs at 1.00 mg protein/mL. This agrees well with our earlier observations (Janssen *et al.* 2020c).

Fig 5. Interferometry images of oat batter liquor (BL) thin films (TFs) at 1.00 mg protein/mL at different time points after applying an excess gas pressure of 200 Pa (eP_g^{200Pa}). D represents the image just before TF rupture.

As drainage progressed, somewhat contradictory results were obtained in the various repetitions of the oat BL TF drainage experiments at eP_g^{200Pa} . In a first set of experiments, TFs ruptured when the A-W interfaces were still dimpled, which implies that TFs ruptured near the Plateau border (the thinnest region of the dimple). However, in a second set of experiments, the dimple contracted progressively, indicating that the A-W interfaces evolved towards a planar shape over time. This was followed by the appearance of white spots dispersed in a black region (**Figs 5C and 5D**). This black region had an average h_{eq} of 12 ± 1 nm just before rupture and most likely represents an inverse lipid bilayer or common black film. Interestingly, these black regions were embedded in a thicker, grey network-like structure (**Fig 5D**). As will be addressed in more detail in section 3.2.2.1, this implies that oat BL TFs at 1.00 mg protein/mL display stratification due to the presence of one or multiple layers of micelles. The presence of micelles in oat BL TFs at 1.00 mg protein/mL is supported by the outcome

of σ measurements at increasing DL bulk concentration (see **supplementary information**), which indicated that the critical micelle concentration was reached at 0.10 mg protein/mL. The above-mentioned white spots were present as distinct circular entities and had average h_{eq} values ranging between 30 and 60 nm (**Fig 5D**). As Marangoni stresses were observed during drainage of oat BL TFs at 1.00 mg protein/mL at eP_g^{200Pa} (**Fig 5A**), these entities were probably not protein aggregates. Interestingly, most of these white spots were located at the rim of the black regions and seemed to be part of the grey network-like structure. Therefore, they may represent liquid expelled during the transition of a TF region in which one layer of micelles is enclosed (i.e. the grey network) to a TF region representing an inverse lipid bilayer or common black film (i.e. the black regions). A similar effect has been observed for sodium dodecyl sulfate (SDS) TFs (Bergeron *et al.* 1992; Zhang *et al.* 2016).

3.2.1.2 Thin film stability

Table 1 shows several parameters characteristic for the drainage experiments carried out with wheat and rye DL TFs at 1.50 and 0.50 mg protein/mL, respectively, as well as of oat BL TFs at 1.00 mg protein/mL. Each of the three TFs ruptured in less than fifteen seconds, both at eP_g^{50Pa} and eP_g^{200Pa} , indicating that they were unstable and that the A-W interfaces surrounding the TF merged rapidly (**Table 1**).

Despite the similar TF stabilities, some notable differences in the drainage dynamics of wheat and rye DL and oat BL TFs were observed. Wheat DL TFs at 1.50 mg protein/mL had lower expansion rates than rye DL and oat BL TFs at 0.50 and 1.00 mg protein/mL, respectively, both at eP_g^{50Pa} and eP_g^{200Pa} (**Table 1**). The expansion rate is influenced by the nature of the stress-boundary conditions (**Fig 1B**) and the pressure difference between the center of the TF and its Plateau border regions, although both phenomena are related (Chatzigiannakis *et al.* 2020).

Table 1. Drainage times, radii at rupture or 180 s after forcibly inducing drainage (i.e. equilibrium radius), expansion rates, bulk viscosities, equilibrium surface tensions, and/or Boussinesq numbers (see **eq 7**) of wheat and rye dough liquor and oat batter liquor thin films at lowered as well as at their native bulk concentrations. Abbreviations: DL, dough liquor; batter liquor, BL; TF, thin film; s^{-1} , unit for the applied shear rate.

	Wheat dough liquor		Rye dough liquor		Oat batter liquor	
Lowered bulk concentration	1.50 mg protein/mL		0.50 mg protein/mL		1.00 mg protein/mL	
DL/BL bulk viscosity (mPa.s)	1.12 ± 0.02		1.09 ± 0.01		1.06 ± 0.01	
DL/BL equilibrium surface tension (mN/m)	46 ± 1		48 ± 1		35 ± 1	
Excess gas pressure (Pa)	<u>50</u>	<u>200</u>	<u>50</u>	<u>200</u>	<u>50</u>	<u>200</u>
TF drainage time (s)	8 ± 1	7 ± 1	9 ± 1	6 ± 1	13 ± 1	8 ± 2
TF Radius at rupture (μm)	156 ± 40	439 ± 28	331 ± 41	702 ± 29	369 ± 42	649 ± 64
TF expansion rate (μm/s)	7 ± 3	30 ± 6	17 ± 4	61 ± 10	12 ± 2	41 ± 12
TF Boussinesq number (-)	1401 ± 322	475 ± 30	873 ± 117	407 ± 19	26 ± 3	15 ± 1
Native bulk concentration	40.1 mg protein/mL				7.0 mg protein/mL	
DL/BL bulk viscosity (mPa.s)	62 (at 1 s ⁻¹) – 6 (at 100 s ⁻¹)				6 (at 1 s ⁻¹) – 2 (at 100 s ⁻¹)	
DL/BL equilibrium surface tension (mN/m)	42 ± 1				32 ± 1	
Excess gas pressure (Pa)	<u>50</u>	<u>200</u>			<u>50</u>	<u>200</u>
TF drainage time (s)	> 180	> 180			> 180	> 180
TF equilibrium radius (μm)	552 ± 70	830 ± 8			583 ± 157	768 ± 99
TF expansion rate (μm/s)	5 ± 1	18 ± 1			3 ± 1	14 ± 1

In addition, A-W interfaces of wheat DL TFs at 1.50 mg protein/mL remained planar both at eP_g^{50Pa} (**supplementary information**) and eP_g^{200Pa} (**Fig 3**), while those surrounding rye DL and oat BL TFs at 0.50 and 1.00 mg protein/mL, respectively, were deformed into a dimple after applying eP_g^{50Pa} (only oat BL TFs) (**supplementary information**) and eP_g^{200Pa} (both rye DL and oat BL TFs) (**Figs 4 and 5**). These two observations suggest that A-W interfaces of wheat DL TFs at 1.50 mg protein/mL have a higher stress carrying capacity than those surrounding rye DL and oat BL TFs at 0.50 and 1.00 mg protein/mL, respectively. That the Bq numbers (**Eq 7**) of wheat DL TFs at 1.50 mg protein/mL at eP_g^{50Pa} and eP_g^{200Pa} were higher than the corresponding Bq numbers of rye DL and oat BL TFs at 0.50 and 1.00 mg protein/mL, respectively, supports this view (**Table 1**). Indeed, a higher Bq number implies a greater contribution of the surface stress-boundary conditions (Hermans *et al.* 2015).

Despite the greater interfacial rheological stress associated with wheat than with rye DL and oat BL stabilized interfaces at lowered bulk concentrations, all these DL TFs still had similar drainage times. Two phenomena may be at the basis for this. First, in most cases drainage occurs faster when the TF radius is lower (Leal, 2004; Janssen and Anderson, 2011). Second, liquid drains more rapidly from between two planar than from between two dimpled A-W interfaces (**Fig 1A**) (Joye *et al.* 1992).

Regardless of the above, wheat and rye DL TFs at 1.50 and 0.50 mg protein/mL, respectively, and oat BL TFs at 1.00 mg protein/mL rapidly reached thicknesses at which their stability mostly depends on the magnitude of Π . That rupture occurred at TF thicknesses of 20 nm for wheat and rye DL TFs and 12 nm for oat BL TFs implies that at this point the combined contributions of P_c and Π_{VDW} were larger than those of Π_{el} and Π_{st} (**Fig 1C**), even though for instance for rye, it was argued earlier that adsorbed proteins indeed engage in intermolecular interactions. It is possible that rupture may have occurred in protein-depleted interfacial regions for which the contributions of Π_{el} and Π_{st} would be too low to counteract those of P_c and Π_{VDW} .

3.2.2 Drainage dynamics of wheat oat dough liquor and oat batter liquor thin films at their native concentrations

3.2.2.1 Thin film structural changes

WHEAT DOUGH LIQUOR

Fig 6 shows interferometry images of wheat DL TFs at their native concentration at different time points after applying eP_g^{200Pa} (**A** to **D**). The 3D pixel intensity plot of **Fig 6D** (i.e. the image taken 180 s after drainage was forcibly induced) is shown in **Fig 6E**. After applying eP_g^{200Pa} , the A-W interfaces surrounding wheat DL TFs at their native concentrations were deformed into a dimple (**Figs 6A**). Wheat DL constituents (visually observed as black and white spots in **Fig 6A**) were observed to migrate to the center of the dimple. As mentioned in section 1, such behavior illustrates that wheat DL constituents seemed to exert Marangoni stresses. This implies that the constituents adsorbed at wheat DL TF A-W interfaces do not form a continuous viscoelastic protein network and that lipids dominate A-W interfaces of wheat DL TFs at their native concentration. Dimple formation was followed by it being 'washed out' from the TF into the Plateau border region (**Figs 1A and 6B**). Once the dimple had been washed out, three distinct regions were observed in wheat DL TFs at their native concentration: a white region (labeled h_1 in **Figs 6C and 6E**), a dark grey region (labeled h_2 in **Figs 6C and 6E**), and black spots embedded in this dark grey region (indicated by white arrows in **Fig 6C**). This is further illustrated by the red/green and dark blue regions, respectively, in the corresponding 3D pixel intensity plots (**Fig 6E**). After 180 s, the white and dark grey regions had average h_{eq} values of 79 ± 4 and 24 ± 2 nm, respectively (**Fig 6D**). Although it was not possible to determine h_{eq} of the black spots embedded in the dark grey region accurately, their thickness is expected to be lower than 24 nm (**Fig 6C**). In addition, two dark black circles representing remnants of the dimple were observed (**Figs 6C, 6D, and 6E**).

Fig 6. Interferometry images of wheat dough liquor (DL) thin films (TFs) at their native concentration at different time points after applying an excess gas pressure of 200 Pa ($eP_g^{200\text{Pa}}$). D represents the image 180 s after applying $eP_g^{200\text{Pa}}$ and is presented in E as a 3D intensity plot, in which lower TF pixel intensities correspond to a lower equivalent thickness (h_{eq}), except for dimple remnants. In the latter, dark blue regions have a greater thickness than the adjacent light blue and white regions because the order of interference is higher in the former.

Over time, dark grey circular domains (dark blue circular regions in **Fig 6E**), which expanded and merged with other similar domains, emerged in the white region (**Figs 6C, 6D, and 6E**). These domains had similar h_{eq} values as the larger dark grey region surrounding the white region. The white regions most likely represent a thick film containing wheat DL constituents separated by two interfacial lipid monolayers. The increase in area of thinner dark grey regions at the expense of thicker white regions (**Figs 6C, 6D, and 6E**) is a phenomenon known as stratification. Stratification occurs when a TF contains at least two layers of supramolecular structures (Nikolov and Wasan, 1989; Kralchevski *et al.* 1990; Bergeron *et al.* 1992; Sonin and Langevin, 1993). Precisely because of this layered structuring, stratifying TFs thin in a stepwise fashion. The Δh_{eq} value of each such step ($\Delta h_{eq,step}$) can be estimated by calculating the sum of (i) the characteristic length (l) of the supramolecular structures confined in the TF and (ii) the Debye length ($1/\kappa$), which is a measure for the distance between different layers (Nikolov and Wasan, 1989):

$$\Delta h_{eq,step} = 2 \left(l + \frac{1}{\kappa} \right) \text{ (Eq 12)}$$

That stratification proceeded through the growth of circular domains indicates that the supramolecular structures in wheat DL TFs are lipid micelles (Nikolov and Wasan, 1989). This agrees well with the observations that Marangoni effects are at play at the interfaces (see section 1) and that wheat DL at its native concentration was above the critical micelle concentration (see **supplementary information**). The radius of lipid micelles largely corresponds to the length of the hydrocarbon chains of the participating lipid molecules (Reiss-Husson and Luzzati, 1964). Linoleic acid is the most abundant FA in wheat DL lipid species (Janssen *et al.* 2020c). The characteristic length of a single linoleic acid molecule was calculated¹ to be 2.04 nm which implies that the radius of linoleic acid micelles would approximately be 4.08 nm. This value agrees well with an h_{eq} value of 3.91 nm reported by Beltramo, Van Hooghten and Vermant (2016) for phospholipid bilayers.

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¹ The characteristic length of a single linoleic acid molecule was calculated by summing up the lengths of the different carbon-carbon bonds, considering bond angles and double bond cis configurations.

The Debye length ($1/\kappa$) can be calculated as follows (Nikolov and Wasan, 1989):

$$\kappa^2 = \frac{4\pi e^2 N_A}{\epsilon k T} C_{el} \text{ (Eq 13)}$$

with e the charge of an electron (4.80×10^{-10} statcoulomb or $\text{cm}^{3/2} \text{g}^{1/2} \text{s}^{-1}$), N_A Avogadro's number ($6.02 \times 10^{23} \text{ mol}^{-1}$), ϵ the dielectric constant of the bulk phase, k the Boltzmann constant ($1.38 \times 10^{-23} \text{ m}^2 \text{kg s}^{-2} \text{K}^{-1}$), T the temperature (298.15 K), and C_{el} the total concentration of electrolytes in the bulk phase (in mol/m^3). Somewhat unfortunately, DL contains a multitude of electrolytes such as endogenously present minerals, sodium chloride, proteins, as well as free FAs and phospholipids (assuming that they occur in their dissociated forms). In addition, the present proteins, free FAs, and phospholipids have a great structural heterogeneity, with molecules varying considerably in molecular weight and charge density. Thus, it is impossible to determine the exact electrolyte concentration (in mol/m^3) in DL and even to estimate it would be at risk of making a fairly large error in calculating the value of $\Delta h_{eq,step}$ (Eq 12). Nonetheless, assuming that the diameter of the lipid micelles in stratifying wheat DL TFs is approximately 4.08 nm and that a lipid monolayer exists at both TF A-W interfaces, it is reasonable to state that the dark blue regions, with an h_{eq} of 24 nm, in Fig 6E contain at least one layer of micelles. Moreover, this would imply that the earlier mentioned black spots, with an estimated h_{eq} lower than 24 nm, embedded in the dark grey region (Fig 6C) represent either one layer of micelles or merely an inverse lipid bilayer or common black film.

OAT BATTER LIQUOR

Fig 7 shows interferometry images of oat BL TFs at their native concentration at different time points after applying eP_g^{200Pa} (A to D). The 3D pixel intensity plot of Fig 7D (i.e. the image taken 180 s after drainage was forcibly induced) is shown in Fig 7E. Interestingly, while surface mobility was observed during drainage of oat BL TFs at lowered bulk concentrations (see section 3.2.1.1), this was not the case during drainage of oat BL TFs at their native concentration.

Stratification or step-wise drainage of supramolecular structures (likely micelles) through the expansion of visually darker and thus thinner circular domains

Fig 7. Interferometry images of oat batter liquor (BL) thin films (TFs) at their native concentration at different time points after applying an excess gas pressure of 200 Pa (eP_g^{200Pa}). D represents the image 180 s after applying eP_g^{200Pa} and is presented in E as a 3D intensity plot, in which lower TF pixel intensities correspond to a lower equivalent thickness (h_{eq}).

This most likely relates to the fact that at higher bulk concentrations diffusion of lipids from the bulk phase to the A-W interface rapidly (in less than 10 ms, which was the maximal temporal resolution of the camera, see section 2.2.4) restored interfacial concentration (and thus σ) gradients, thereby suppressing otherwise occurring Marangoni stresses. Similar results have been observed for surfactant films at concentrations exceeding the critical micelle concentration (Radoev, Dimitrov and Ivanov, 1974; Bhamla Saad, Chai, Alvarez-Valenzuela, Tajuelo and Fuller, 2017). It thus seems that A-W interfaces of oat BL TFs at their native concentration consist of densely-packed lipid monolayers. The low equilibrium σ of A-W interfaces stabilized by constituents in oat BL at its native concentration (**Table 1**) supports this.

For oat BL TFs at eP_g^{200Pa} at their native concentration, three distinct regions were observed after the dimple had washed out: small white spots (**Fig 7C, 7D, and 7E**), light grey regions (labeled h_1 in **Figs 7D and 7E**), and dark grey regions (labeled h_2 in **Figs 7D and 7E**). The white spots had h_{eq} values ranging from 20 to 60 nm (**Fig 7D**) and appeared as discrete dark red entities in the corresponding 3D pixel intensity plots (**Fig 7E**). Such spots have been referred to as ‘satellite fluid pockets’ or ‘Rayleigh-type instabilities’ (Bergeron *et al.* 1992) and nanoscopic mesas or terraces (Zhang *et al.* 2016)² and are believed to originate from accumulation of surface active moieties at the rim of the expanding domains. This rim breaks apart as a result of local differences in Π_{sup} (**Fig 1C**) as well as in curvature and thus in P_L between its interior and exterior (Zhang *et al.* 2016). At eP_g^{200Pa} , the light and dark grey regions had average h_{eq} values of 18 ± 2 and 15 ± 2 nm, respectively (**Figs 7D**), which corresponded to high (light red) and low (green) TF pixel intensities in **Fig 7E**.

That thinner circular regions expanded at the expense of thicker regions shows that oat BL TFs at their native concentration - as did wheat DL TFs - exhibited stratification due to layering of micelles. This is in line with the presence of a lipid interfacial monolayer (see section 3.2.1.1) and with σ remaining

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² Bergeron *et al.* (1992) and Zhang *et al.* (2016) compared these instabilities with the rim of a volcano crater that disintegrates into discrete pockets.

constant already at an oat BL bulk concentration of 0.10 mg protein/mL (see **supplementary information**). A similar reasoning as above for wheat DL TFs results in an estimated oat BL TF lipid micelle diameter of approximately 4.08 nm. Hence, assuming that there is a lipid monolayer at both A-W interfaces, it seems likely that the light red planar regions, with an h_{eq} of 18 nm, in **Fig 7E** may contain at least one layer of micelles, whilst the green regions, with an h_{eq} of 15 nm, in **Fig 7E** may represent merely an inverse lipid bilayer or common black film.

3.2.2.2 Thin film stability

Neither at eP_g^{50Pa} nor at eP_g^{200Pa} did wheat DL and oat BL TFs at their native concentrations rupture after 180 s (**Table 1**). Some additional drainage experiments at eP_g^{50Pa} showed that neither of the TFs ruptured even after 600 s. Both wheat DL and oat BL TFs at their native concentrations were thus very stable. Despite similar TF stabilities, some notable differences in the wheat DL and oat BL TF drainage dynamics were observed.

Although wheat DL and oat BL TFs at their native concentrations expanded to a similar $R_{TF,eq}$ over time after applying eP_g^{200Pa} , oat BL TFs expanded slower than wheat DL TFs, despite the higher η_b and $P_{L,bw}$ (**Eq 3**) of the latter (**Table 1**). This discrepancy may to some extent relate to Marangoni stresses during drainage of the latter but not during that of the former. Indeed, Marangoni stresses may create a line tension gradient between the rim of the dimple (higher concentration of surface-active constituents) and the TF Plateau border region (lower concentration of surface-active constituents) (Ivanov and Kralchevsky, 1997). Such gradient is expected to promote the diffusion of constituents towards the Plateau border region, which may increase the TF expansion rate.

Another notable difference in wheat and oat BL TF drainage dynamics was that, irrespective of the applied eP_g , the dimple of a wheat DL TF had a greater thickness at its center than an oat BL TF dimple (**Figs 6A and 7A**). This implies a higher stress-carrying capacity for wheat DL TF than for oat BL TF A-W interfaces, which is in line with the observation that surface mobility by lipids, and thus the occurrence of

Marangoni forces, was only observed during drainage of the former. That interfaces with greater stress-carrying capacities result in larger dimples has also been noted elsewhere (Dai and Leal, 2008; Janssen and Anderson, 2011).

Interestingly, wheat DL TF dimples washed out later (51 ± 2 s after applying $eP_g^{200\text{Pa}}$) than those of oat BL TFs (29 ± 4 s after applying $eP_g^{200\text{Pa}}$) (**Figs 6B** and **7B**). In this regard, Joye *et al.* (1994) for a TF consisting of sodium dodecyl sulfate (SDS) or SDS-1- dodecanol (100:1) demonstrated that the magnitude of the interfacial shear viscosity ($|\eta_s|$) largely determines the moment at which dimpled A-W interfaces are washed out from the TF. It would thus be of interest to assess the interfacial shear rheology of wheat DL and oat BL at their native concentrations. However, the exact mechanism driving the wash-out of dimples from TFs is still not well-understood. Finally, it remains to be investigated whether dimpling would even occur in bread making as gas cells in dough expand quite slowly and the TFs separating them may not experience an abrupt P_H increase.

Earlier it was shown that wheat DL and oat BL TFs at their native concentrations display stratification behavior. Stratifying TFs have high stability as they are thermodynamically metastable. Indeed, each number (> 1) of micellar layers creates an additional metastable state in the Π isotherm (Nikolov and Wasan, 1989; Bergeron and Radke, 1992) (**Fig 1C**). The magnitude of Π_{sup} at these metastable TF thicknesses generally increases as the number of micellar layers decreases and can reach values up to 200 Pa (Zhang and Sharma, 2018) or even 1,000 Pa (Basheva *et al.* 2007). However, the magnitude of Π_{sup} at such metastable thicknesses is system-specific and amongst others depends on the molecular structure and concentration of the involved surfactants (Basheva *et al.* 2007; Zhang *et al.* 2016). Nonetheless, earlier it was mentioned that gas cells in bread dough at the end of fermentation may experience $P_{L, gas\ cell}$ values lower than 200 Pa. It thus seems reasonable to assume that at this stage at least some of the gas cells which are separated only by a TF are metastable and therefore temporarily prevented from coalescing. Furthermore, even at low TF thicknesses (< 30 nm) wheat DL and oat BL TFs at their native concentrations

remained stable. This implies that there may have been a contribution of Π_{el} and/or Π_{st} to the total Π of said TFs, possibly originating from intermolecular interactions occurring between the lipid monolayers at both TF A-W interfaces.

3.3 Implications for bread making research

Based on the above, it can be concluded that the stability of gas cells during bread dough fermentation and early baking likely not only depends on the characteristics of their A-W interfaces, but also on those of the TFs separating them. Indeed, the use of a pressure-controlled dynamic TF balance can provide beyond state-of-the-art insights into the potential of DL constituents to stabilize gas cells in bread making. Moreover, the present study demonstrates the importance of studying the drainage dynamics of DL TFs at native rather than at lowered concentrations, as the bulk and interfacial stabilization mechanisms in both cases were substantially different. Wheat DL and oat BL constituents when assessed at their native concentration may contribute substantially to stabilizing gas cells in dough and thus likely impact bread loaf volume and crumb structure. That wheat breads have a considerably higher loaf volume than oat breads (Janssen *et al.* 2018) is mostly due to the viscoelastic gluten network which by displaying strain hardening acts as the primary gas cell stabilizing force (van Vliet *et al.* 1992). Thus, assuming that oat batter aqueous phase constituents contribute to gas cell stability in oat bread making, they cannot match the efficiency of the combined contributions of the gluten network and dough aqueous phase constituents in terms of stabilizing gas cells in wheat bread making.

4. Conclusions

The role of TF drainage dynamics in determining gas cell stability in bread making has so far received little attention. Most available experimental studies dealing with the A-W interfacial properties of DL/BL constituents have thus far been carried out at concentrations much below those in real

dough/batter systems. In the present work, we studied DL/BL TFs at their native concentrations, or at least at concentrations as high as possible without clogging the microfluidic chips. First, DL/BL TFs at their native concentrations expanded slower and to a larger radius than those at lowered concentrations. This was related to differences in the nature of the stress-boundary conditions, η_b (although at both concentrations NSPs were enzymatically hydrolyzed) and pressure differences between the TF center and Plateau border, which are interrelated phenomena. Weakly viscoelastic A-W interfacial layers dominated by proteins were characteristic of rye DL TFs at 0.50 mg protein/mL (**Fig 8 C1**). Wheat DL TFs at relatively low concentrations (1.50 mg protein/mL) were characterized by weakly viscoelastic mixed protein-lipid A-W interfaces (**Fig 8 A1**). In contrast, wheat DL TF A-W interfaces at their native concentration seemed to be dominated by lipids which then generated Marangoni stresses upon formation of a dimpled film (**Fig 8 A2**). Whilst dimples and likely thus the associated convective effects were also observed for oat BL TFs, both at 1.00 mg protein/mL (**Fig 8 B1**) and at native concentration (**Fig 8 B2**), surface mobility by lipids was only observed at the former concentration. Second, the dimpling for wheat DL and oat BL TFs at native concentration likely also contributed to their reduced liquid drainage rates. Finally, DL/BL TFs at their native concentration seemed to be stable at low TF thicknesses, due to a combined effect of stratification and intermolecular repulsive forces, whilst at lowered bulk concentrations they were not. We conclude that wheat DL and oat BL constituents when assessed at their native concentration might contribute substantially to stabilizing gas cells in dough and thus likely also to bread loaf volume and crumb structure.

Fig 8. Schematic overview of the structure of wheat dough liquor (DL) (**A1**), oat batter liquor (BL) (**B1**), and rye DL (**C1**) thin films (TFs) at 1.50, 1.00, and 0.50 mg dry matter protein/mL, respectively, as well as of wheat DL (**A2**) and oat (**B2**) BL TFs at their native concentrations (i.e. at concentrations equal to those occurring in the respective supernatant after ultracentrifugation). Rye DL TFs at their native concentration could not be monitored because rye DL constituents formed large aggregates in the microchip channels which prevented drainage to occur freely. Abbreviations: Π_{sup} , disjoining pressure contribution from oscillatory structural forces; Π_{el} , disjoining pressure contribution from electrostatic repulsive forces; Π_{st} , disjoining pressure contribution from steric repulsive forces; A-W, air-water.

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