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RESEARCH ARTICLE

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Microscopic analysis of gluten network development under shear load—combining confocal laser scanning microscopy with rheometry

Leonhard Maria Vidal[1] | Hans Ewigmann[1] | Clemens Schuster[2] | Thekla Alpers[1] | Katharina Anne Scherf[3] | Mario Jekle[4] | Thomas Becker[1]

[1]Research Group Cereal Technology and Process Engineering, Institute of Brewing and Beverage Technology, Technical University of Munich, Freising, Germany

[2]Leibniz Institute for Food Systems Biology at the Technical University of Munich, Freising, Germany

[3]Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

[4]Department of Plant-Based Foods, Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

Correspondence
Leonhard Maria Vidal, Research Group Cereal Technology and Process Engineering, Institute of Brewing and Beverage Technology, Technical University of Munich, D-85354 Freising, Germany.
Email: leonhard.vidal@tum.de

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Abstract

A comprehensive in-situ analysis of the developing gluten network during kneading is still a gap in cereal science. With an in-line microscale shear kneading and measuring setup in a conventional rheometer, a first step was taken in previous works toward fully comprehensible gluten network development evaluation. In this work, this setup was extended by an in-situ optical analysis of the evolving gluten network. By connecting a laser scanning microscope with a conventional rheometer, the evaluation of the rheological and optical protein network evolution was possible. An image processing tool for analyzing the protein network was applied for evaluating the gluten network development in a wheat dough during the shear kneading process. This network evaluation was possible without interruption or invasive sample transfer comparing it to former approaches. The shear kneading system was able to produce a fully developed dough matrix within 125% of the reference dough development time in a classical kneader. The calculated network connectivity values from frequency testing ranged over all samples was in good agreement with traditional kneaded wheat dough just over peak consistency.

KEYWORDS
confocal laser scanning microscopy, gluten network, rheology, wheat flour dough

1 | INTRODUCTION

Wheat flour dough is characterized by a highly crosslinked and widespread protein network (Jekle & Becker, 2011a). The three-dimensional network enables the matrix to retain gas and is therefore, together with the heat-induced starch gelatinization, responsible for the achievement of high loaf volumes and appealing crumb structures after baking (Dowell et al., 2008; Hrušková et al., 2006; Wikstrom & Bohlin, 1996). This protein network evolves under hydration and input of mechanical energy (Jekle & Becker, 2015). The mechanical energy input in classical kneaders comprises tension, shear and/or compression (Connelly & Kokini, 2006, 2007; Tietze et al., 2017). The specific mechanical energy is crucial for the development of the desired network and is transferred from the kneading elements of the mixer to the material. The basis for most baked goods is the formation of an optimal developed gluten phase in the dough matrix. The optimum

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consistency of a dough is achieved when the dough best withstands the deformation caused by the kneading geometry and is therefore the peak of dough consistency (Parenti et al., 2021). At this stage of optimal dough development, the combination of covalent (disulfide) and non-covalent (e.g., H-bond, Van-der-Waals) bonds of the polymeric protein network is responsible for the specific mechanical behavior of the dough. The protein network consists mainly of two main fractions, glutenins and gliadins, the storage proteins of the grain which consist of high proportions of the amino acids glutamine (32%–53%) and proline (11%–29%) (Wieser et al., 2022). First, glutenins, represented by high and low molecular weight glutenin subunits (HMW-GS and LMW-GS), are polymeric. And second, gliadins are represented by α-, γ-, $\omega_{1,2}$-, and ω_5-gliadin, which are monomeric (Lindsay & Skerritt, 1999). The glutenins and gliadins show a wide variety of amino acid compositions and differ in their ability to bind other proteins and other flour constituents (Wieser et al., 2022). Besides these protein fractions, albumins and globulins are also present in wheat flour. These non-gluten proteins are mainly monomeric, but both albumins and globulins tend to form polymers by forming interchain disulfide bonds as well, but they make no significant contribution to the achievable loaf volume or baking quality of wheat flours (Tomić et al., 2015). At the beginning of the dough kneading process, the combination of mechanical energy input and the hydration of the flour particles causes glutenins and gliadins to crosslink and develop a continuous protein network. This network is known as the continuous gluten phase (Delcour & Hoseney, 2010; Jekle & Becker, 2011a; McCann & Day, 2013). Starch granules are also embedded in the gluten phase, which promote internal friction during kneading. Regardless of their shape and size, the surface functionality of the starch granules can be considered as a universal factor influencing the resulting network, since their surface contributes to the interface between the particle and the gluten phase (Brandner et al., 2021). Therefore, the resulting dough matrix depends both on the gluten attributes as well as on the starch protein interactions.

The kneading process is difficult to reproduce due to its complexity and the action of several forces. Therefore, it is hardly possible to completely trace the development of the dough matrix in-line analytically. In addition to the limitation due to complexity, an interruption of the process and sample transfer of the kneaded sample to other equipment (e.g., a microscope) is mandatory. These interruptions are the state-of-the-art to evaluate the dough matrix at different stages of development on a microstructural level. To isolate energy transfer from the complex kneading geometries of most commercial kneaders, Vidal et al. (2022) developed a microscale kneading process that uses only shear to develop a dough matrix. Analyzing the evolving protein network in gluten-starch systems and wheat flour doughs on a microscale using a confocal laser scanning microscope (CLSM) is already state-of-the-art in cereal science. The method established by Jekle and Becker (2011b) can be utilized as a tool for protein quantification. Additionally, the protein network analysis (PNA) developed by Bernklau et al. (2016) allows the identification of absolute morphological attributes such as total number of junctions (TNoJ) or the branching rate as well as end-point rate and lacunarity, and can precisely quantify and characterize the development stage of a gluten network. With the aforementioned tools for in-line production of dough in a rheometer and the possibility to precisely calculate the development stages of the network by means of microscopic analysis, the basis for a complete analysis of the network development would be given. Thus, by combining a rheometer with a microscale kneading technique and a CLSM in one unit, the development process of the resulting dough matrix could be studied in greater detail. As shown by Dutta et al. (2013) the inline measurement of network evolution in collagen polymerization with such a rheometer-CLSM coupling is possible and may be applicable for wheat dough network evaluation.

The aim of the study was to develop a combined kneading and microscopic analysis tool in a rheometer (Rheo/CLSM) and to evaluate the mechanical behavior simultaneously with the microstructural evolution of the emerging dough matrix. During kneading, the matrix development and the resulting rheological properties of the dough are influenced by the protein content and the gliadin and glutenin subunits of the flour. To gain more knowledge on these influences at the microstructural level, the tool should be able to precisely determine these intercorrelations from network attributes to flour composition. To investigate the influence of flour protein composition on the achievable network characteristics, a variation of five different wheat flours was analyzed. Without a sample transfer to other devices, the system provides all necessary data to determine the stage of dough development at the rheological and microscopic level and their dependency on the flour composition in-situ. As shown in previous works, the shear induced changes in polymeric matters can be further evaluated by the combination of rheology and microscopy in a combined analysis tool (Gagnon et al., 2020). With this aim, the new method can produce wheat flour doughs in a rheometer with additional microscopic analysis on a very small scale.

2 | MATERIALS AND METHODS

2.1 | Analysis of the wheat flour

For all experiments, commercial wheat flour type 0 and type 00 provided by Rieper AG, Vintl, Italy was used. The composition (see Table 1) has been previously analyzed (Vidal et al., 2023).

To determine the required dough development time (DDT) and water addition, the flours were analyzed according to AACCi 54–70.01 by Vidal et al. (2023) and the resulting specific kneading times derived from the DDT are listed in Table 2. The specific kneading times for further analysis were chosen to get a good overview of the different development stages of the emerging dough matrix.

2.1.1 | Analysis of flour protein composition

Flour proteins were analyzed according to Wieser et al. (1998). Albumins and globulins, gliadins and glutenins were extracted from flour in three steps using a Na_2HPO_4/KH_2PO_4 buffer (0.67 mol/L, pH = 7.6)

Flour	S1	S2	S3	S4	S5
Protein [g/100 g]	14.86 ± 0.06	14.29 ± 0.03	14.22 ± 0.01	11.79 ± 0.03	11.77 ± 0.05

TABLE 1 Flour samples and their protein content.

Flour	50% DDT [s]	100% DDT [s]	125% DDT [s]	150% DDT [s]	600 s
S1	151	302	377	453	600
S2	86	172	215	258	600
S3	137	274	342	411	600
S4	57	114	142	171	600
S5	64	128	160	192	600

Note: Specific analysis points derived from the DDT. A reference kneading time of 600 s for undocked comparison of kneading influence of the network evolution from DDT.

TABLE 2 Determined dough development times (DDT) in a DoughLAB for all flours with optimum water dosage.

containing 0.4 mol/L NaCl, 60% aqueous ethanol (v/v) and a mixture of TRIS/HCl buffer (0.1 mol/L, pH = 7.6) and 1-propanol (50/50; v/v) containing 2 mol/L urea and 10 mg/mL dithiothreitol. RP-HPLC analysis was performed on a Jasco XLC HPLC (Jasco Deutschland GmbH, Pfungstadt, Germany) as described by Schuster et al. (2022) of the injection volume was 20 µL for albumins and globulins, 10 µL for gliadins and 20 µL for glutenins. All determinations were performed in triplicate.

2.2 | Standard wheat dough

For the shear rheological measurements of classically kneaded dough, the specific amount for each flour (corrected to 14% moisture) and demineralized water were kneaded at 63 rpm using a z kneader DoughLAB (Perten Instruments AB, Hägersten, Sewden), equipped with a 50 g mixing bowl. To stain the samples for confocal laser scanning microscopy, 5 mL of bulk water was replaced by a rhodamine B solution (Merck KGaA, Darmstadt, Germany, 0.01 g/100 mL water). All measurements were performed in triplicate. After kneading, a small piece of dough was placed in the CLSM and analyzed according to Section 2.2.2.

2.2.1 | Small-scale deformation analysis

To analyze the rheological property, a frequency sweep within the linear viscoelastic limit was performed on a rheometer type MCR502 (Anton Paar, Osterfildern, Germany). For the frequency sweep, the measurement frequency was varied from 0.1 to 50 Hz at a deformation of 0.05%. The complex modulus G^* was analyzed according to the power law equation (Gabriele et al., 2001):

$$G^*(\omega) = A_f * \omega^{\frac{1}{z}},$$

where A_f (Pa $s^{1/z}$) describes the gel strength and z (-) describes the network elements' connectivity.

2.2.2 | Microstructural analysis

For optical analysis, the dough samples were stained by adding rhodamine B dye into the bulk water to visualize the contained proteins. After kneading, 2 g of the dough sample of each flour was transferred to an object carrier with a cylindrical notch (Ø 18 mm, height 7 mm) and sealed with a cover glass. After 10 min of resting time for dough relaxation, the samples were analyzed by an eclipse Ti-U inverted microscope with an e-C1 plus confocal system (Nikon GmbH, Düsseldorf, Germany) using a laser with a wavelength of 543 nm for excitation, the emission was detected at 590 nm with a 50 nm bypass filter. Four different images were taken of each dough sample with a resolution of 1024 × 1024 pixel and a size of 686 × 686 µm (for 20× magnification). The dough samples were produced in triplicate; therefore, 12 images were analyzed for one flour type. The analysis was performed according to Bernklau et al. (2016).

2.2.3 | Image processing and analysis

The software-based analysis of CLSM images was performed by AngioTool64 version 0.6a (National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA) (Zudaire et al., 2011). The AngioTool was applied on CLSM images of gluten network according to the method of Bernklau et al. (2016) (see Figure 1).

2.3 | Shear-kneading dough production

To produce shear kneaded dough in the MCR502 rheometer (Anton Paar, Ostfildern, Germany), a plane plate-cylinder geometry setup with 25 mm diameter of the upper plate geometry and 25.1 mm inner diameter of the cylinder was used. For all experiments, 192 mg of flour and demineralized water stained with rhodamine B (Merck KGaA, Darmstadt, Germany), 0.01 g/100 mL water were kneaded in the rheometer according to Vidal et al. (2022). The sample preparation is shown in Figure 2. After setting up the application cylinder, the

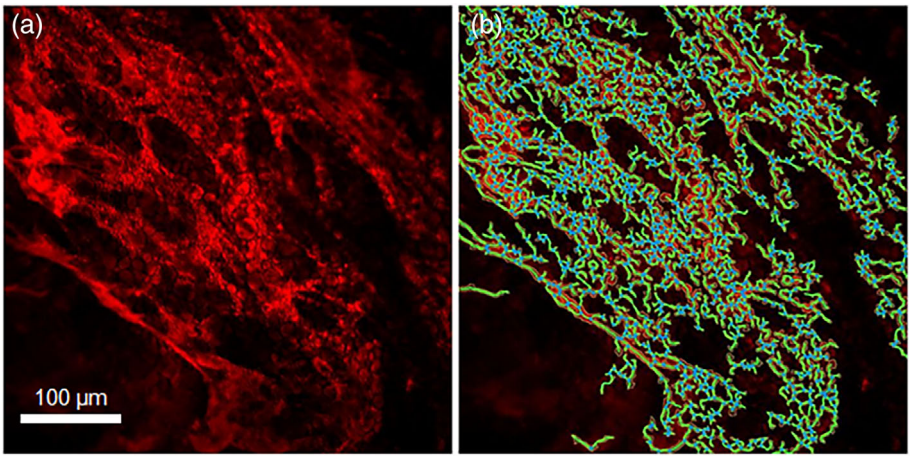

FIGURE 1 (a) Confocal laser scanning microscope (CLSM) image (1024 × 1024 pixels, 686 × 686 µm) of dough from flour S3 after a kneading time of 411 s (150% DDT) using Rheo/CLSM coupling; (b) AngioTool evaluation of image (a) with protein strands (green) and linkage points (blue) drawn in.

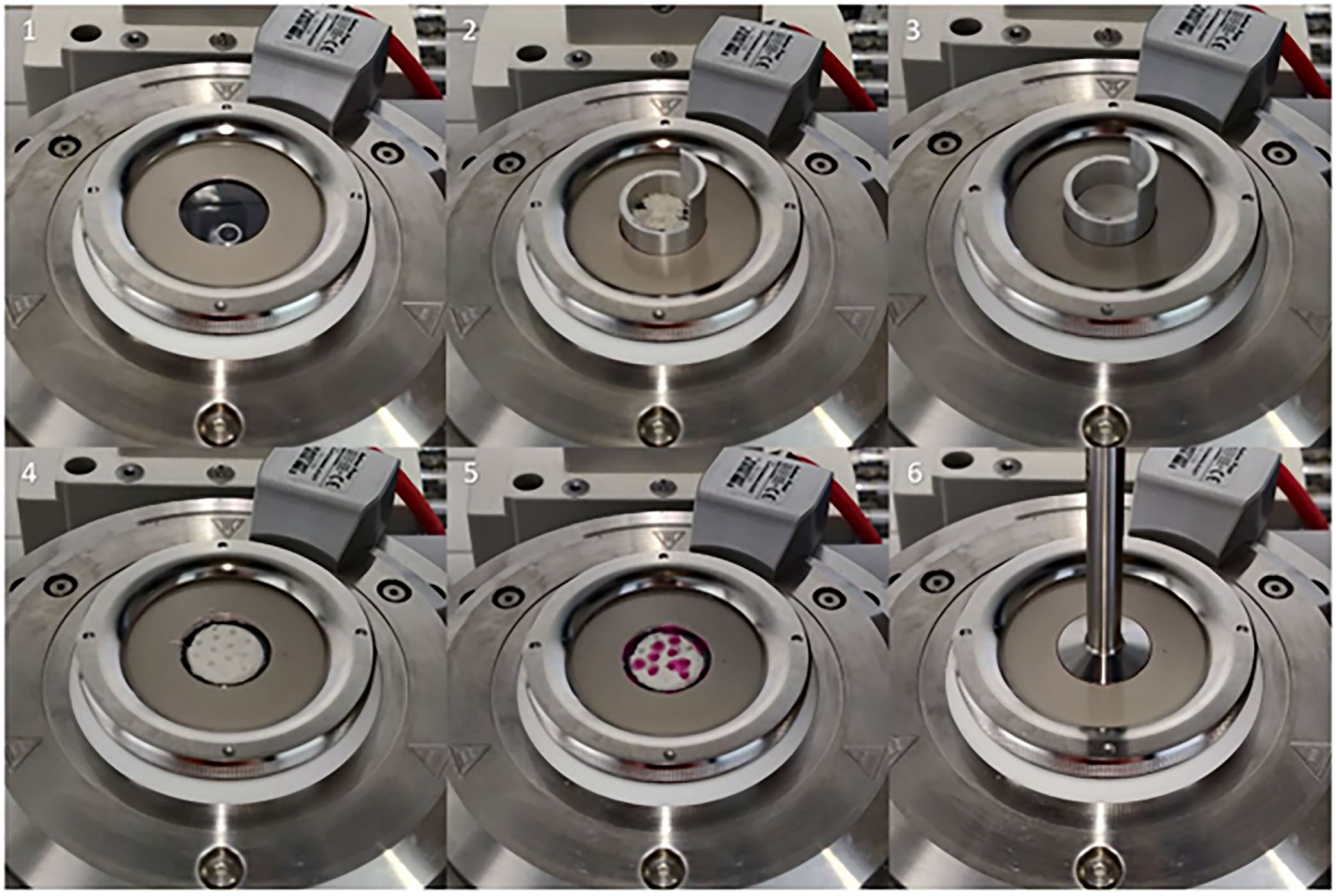

FIGURE 2 Setup of the coupling between rheometer and confocal laser scanning microscope. Process of sampling and setting the upper rheometer geometry with flour S3.

sample was added and smoothed with a smooth stamp. Subsequently, 12 wells were formed with a customized stamp, into which the rhodamine B solution was added dropwise. After adding the stained water, the kneading process started. At specific times, the kneading process was interrupted to capture images with the CLSM (see Table 2). The images were then analyzed according to Section 2.2.2.

2.4 | Statistical analysis

Results were evaluated statistically with Origin(Pro), Version Version 2022. (OriginLab Corporation, Northampton, MA, USA), with Pearson Correlation coefficient calculation. All values are represented with the standard error of the mean (SEM).

3 | RESULTS AND DISCUSSION

3.1 | Composition of the wheat flours

The results of the HPLC analysis in Figure 3 showed that with a lower total protein content of the wheat flour samples, higher quantities of albumin/globulin fractions occurred simultaneously. Tomić et al. detected the content of albumin fractions also in a range from 2.64% to 17.50% of total protein (Tomić et al., 2015). Taking into account, that the albumin and globulin fractions were determined together the observations are in good agreement with the literature (DuPont et al., 2005; Singh & Skerritt, 2001; Tomić et al., 2015). The results also show that the glutenin content for the analyzed flours increases with the total amount of protein measured. Only S4 does not fit these results and has the lowest value for glutenin from HPLC analysis with only 24.0%. The achievable protein composition during breeding is connected to fertilization and varying weather conditions and therefore such differences are not unusual (Edwards et al., 2007). Since the connection of achievable dough volume and total protein content is also linked to protein composition (Skerritt et al., 1999), the flour samples give a good overview of the actual processed flours in the baking industry. To evaluate the network development and rheological behavior of the kneaded dough samples in-situ with a combination of rheology and laser scanning microscopy, the results of the HPLC analysis give the possibility to elucidate these processes precisely. In Table 3, the difference between the analyzed flour is even more pronounced especially for the LMW-GS. As stated by Sisson et al. and Edwards et al., the variation in gluten strength found among LMW-GS and HMW-GS groupings suggest that the presence of specific allelic patterns can, but do not guarantee, a specific level of baking performance (Edwards et al., 2007; Sissons et al., 2005). The variation in total protein and gluten protein subunits gives a good basis for the microstructural evaluation of the network forming processes.

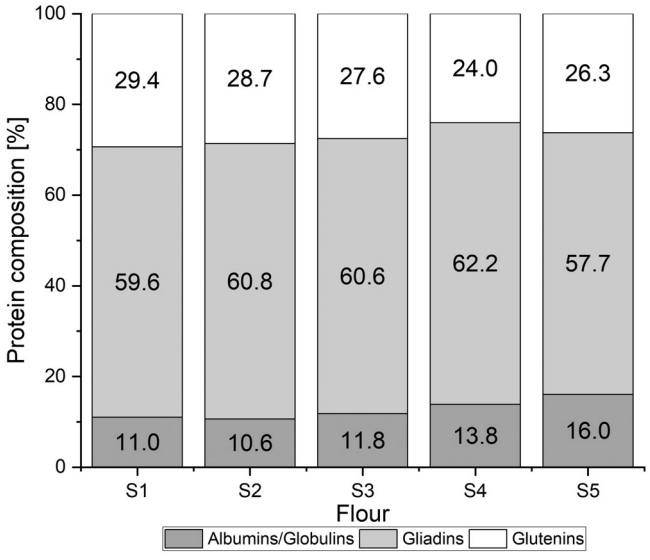

FIGURE 3 Protein composition of the flours divided into albumins/globulins, gliadins and glutenins, given as percentages relative to the sum of total extractable protein (modified Osborne fractionation and RP-HPLC analysis).

3.2 | Microscopic network analysis

Figure 4 shows CLSM images taken during the ongoing kneading process and the network evolution for both classical and shear-kneading processes. As a result of hydration and crosslinking processes in combination with mechanical energy input, the protein strands and nodes emerge from clusters of non-connected flour aggregates to interconnected protein filaments (Schiedt et al., 2013). The network develops from not connected flour particle aggregates at 137 s, consisting of starch, fiber, and protein, to an interconnected protein network at 342 s, with embedded starch and other components. The emerging network consists of the mostly elastic polymeric glutenin network and gliadins are embedded within this matrix. The combination of these two interacting protein fractions gives the dough matrix its unique viscoelastic attributes. The gluten network consists, on the one hand, of covalent disulfide bonds, which contribute to the plasticity of dough. On the other hand, it contains non covalent interactions, especially intra- and intermolecular hydrogen bonds, which are mainly responsible for determining the elasticity (Belton, 1999; Brandner et al., 2018). The resulting network development continues up to a certain point after which the applied load causes a network breakdown. As can be seen in the classically kneaded dough from 342 to 411 s and further on in Figure 4, the network configuration changes to a fibrillary appearance, which results in less strong but highly connected protein strands. This breakdown is caused mainly by three different processes, disentanglement, chain orientation, and bond rupture (Singh & MacRitchie, 2001). With ongoing kneading and network breakdown, the quantity of bigger protein clusters changes (Don et al., 2003). At the end of the performed kneading trials, the over-kneaded sample at 600 clearly shows destroyed and rough strands, where bigger aggregates are separated due to the network breakdown. Looking at

Flour	ω_5-gliadin	$\omega_{1,2}$-gliadin	α-gliadin	γ-gliadin	HMW-GS	LMW-GS
S1	3.9	3.8	31.2	20.8	8.6	19.9
S2	3.6	3.8	33.0	20.4	8.6	19.3
S3	3.9	4.0	32.2	20.6	8.1	18.7
S4	3.9	3.9	32.3	22.1	5.9	17.5
S5	3.3	3.5	29.6	21.4	6.6	19.0

TABLE 3 Differentiation of gliadin and glutenin subunits given as percentages relative to the sum of total extractable protein (modified Osborne fractionation and RP-HPLC analysis).

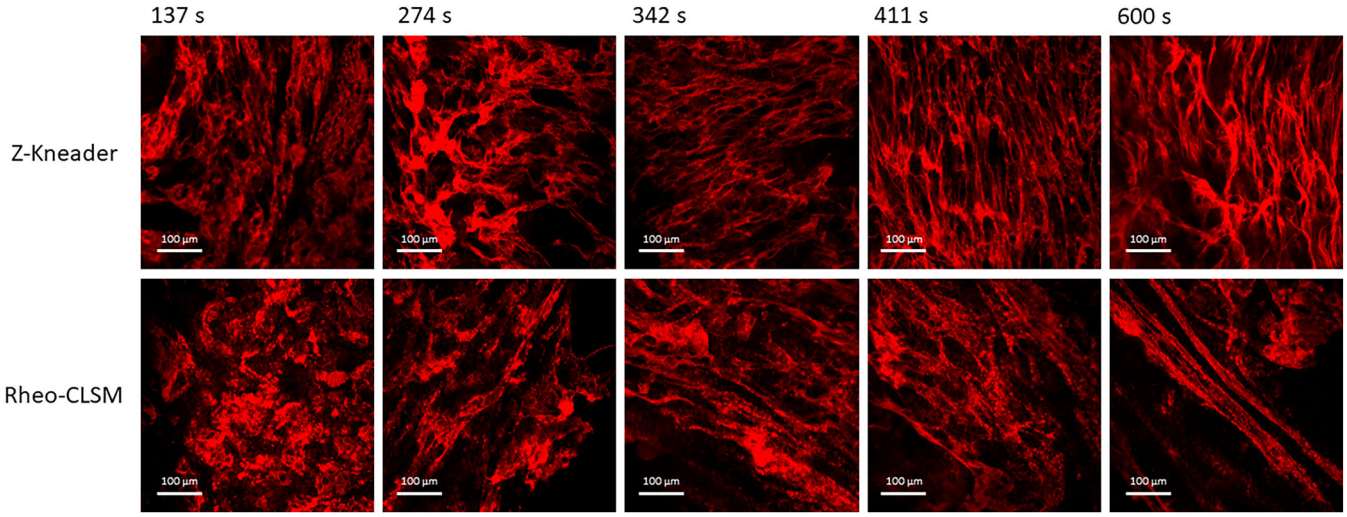

FIGURE 4 Confocal laser scanning microscope (CLSM) images of the evolving protein network of wheat flour S3 at the specific kneading times. Comparison of classically produced dough externally analyzed with a CLSM with shear-kneaded in-line microscopy images during the dough network evolution.

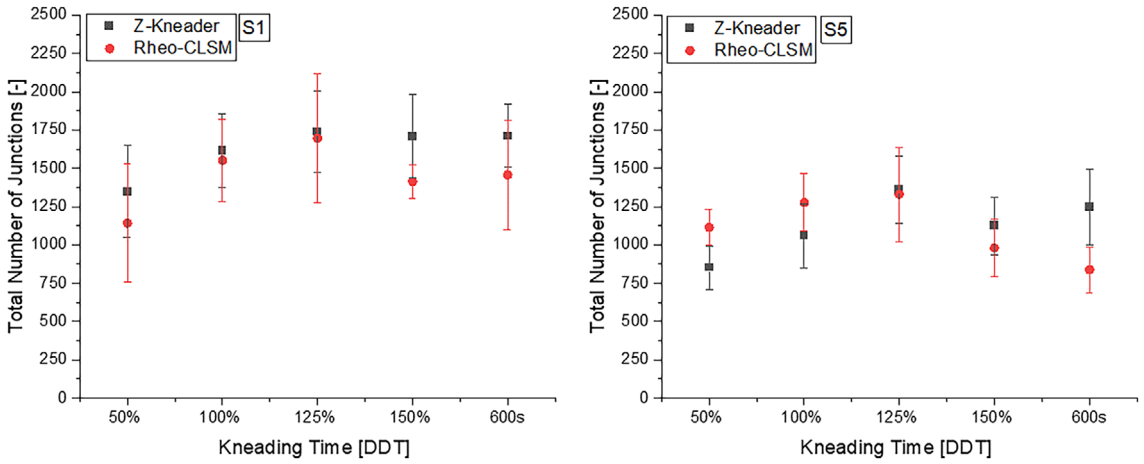

FIGURE 5 Total number of junctions for flour S1 (left) and S5 (right) from the protein network analysis of the confocal laser scanning microscope images taken from standard kneaded wheat dough and shear-kneaded samples. For each sample, three doughs were kneaded and for each time, three images were taken and analyzed.

the shear-kneaded sample, a similar behavior can be observed. The shear-induced destruction of the protein network and the development of bigger separated strands is visible at 600 s kneading time in the rheometer. To further investigate the network evolution, the PNA was performed.

A distinct increase in interconnections as well as protein network breakdown can be seen in Figure 5 by the detected number of nodes when evaluating the CLSM recordings for both flours. The standard deviation derives from threefold dough sample preparation with three images per sample taken for each time, which results in nine images per analysis time. The peak TNoJ indicates the maximum interconnected state of the polymeric matrix in the dough which is influenced by protein content and kneading time (Bernklau et al., 2016; Jekle & Becker, 2015). For both wheat dough samples, representing the highest (S1) and lowest (S5) protein contents of the used wheat flour, the peak can be found at by 125% DDT which can be explained by the ongoing overmixing and therefore changes in the structural appearance of the network (Schiedt et al., 2013). The shear-kneaded samples also showed the optimum development stage close to the DDT determined by the torque recording mixer. S5, with lower protein content, also results in lower TNoJ which is in good agreement for glutenin macro polymer (GMP) formation in the literature (Don et al., 2003; Wang et al., 2019). The less GMP formation, which is built during kneading, can therefore be explained due to lower gliadin and glutenin content (Lindsay & Skerritt, 1999). The breakdown behavior depends on the strength of the protein–protein interactions in the dough and has shown the potential to give an indication on protein content and composition characteristics in wheat (Pritchard &

Brock, 1994). The observed extent of network degradation is different for all the flours studied, since the observed increase in viscosity during mixing, followed by a decrease during overmixing, is caused by the increase in the molecular weight of the polymer as influenced by the gluten content (Skerritt et al., 1999). The gluten content-dependent behavior could also be observed in the CLSM results of this study. Especially the HMW-GS showed a strong Pearson correlation of 0.94 ($p < .05$) with the resulting TNoJ. Within gliadins, the γ-gliadins showed a negative correlation with the TNoJ with −0.87 ($p < .05$). These two gluten protein types also showed strong correlations to the DDTs of the flour samples. Pritchard and Brock (1994) found that the weight of gel protein in flour, and therefore total protein content, was poorly correlated with loaf volume and that correlations between breakdown rate and loaf volume were somewhat better: low loaf volumes were associated with higher rates of breakdown (Skerritt et al., 1999). Loaf volume for the flours was investigated in Vidal et al., underlining these findings for the less strong flours used (Vidal et al., 2023).

3.3 | Rheological network analysis

As shown in Figure 6, the results from standard analysis and in-situ inline testing of the kneaded dough samples were in good agreement and the rheological behavior was comparable. This kneading behavior was previously investigated by Vidal et al. (2022). The reduction in the number of connections due to the collapse of the network (as seen in Figure 4) also reduces the connectivity number z for all samples, which is due to the extended kneading times. Therefore, the value of z as the number of rheological units correlated with one another in the three-dimensional structure drops after at 125% DDT to lower values for all flour samples. This drop could be explained by the breaking of disulfide bonds and a release of free water into the matrix (Haraszi et al., 2008). The lower protein flour S5 shows lower z values for the shear-kneaded sample. The fitted z values strongly correlate with the LMW-GS with a Pearson coefficient of .89 ($p < .05$). The influence of LMW-GS groupings, in this case shown by the connectivity z, can but must not guarantee a higher level of gluten strength (Edwards et al., 2007). Gliadins showed a medium strong negative correlation coefficient of −.77 ($p < .5$) with the z values. Since gliadins are not polymeric, a higher content results in lower glutenin content and therefore less available network building proteins (Costa et al., 2013). During kneading, the quantity of large protein clusters changes (Don et al., 2003). Thus, the network configuration changes to a fibrillary appearance with less strong but highly connected protein strands as shown in the PNA and CLSM images (Appendix).

4 | CONCLUSION

With the developed setup the comparability of shear-kneaded to classically (in a conventional Z-kneader) produced wheat flour dough along the kneading process could be demonstrated. The shear kneading and microscopic setup in a conventional rheometer represents a useful tool to analyze structural formation reactions of the gluten phase. Furthermore, the system provides a controlled energy input method to investigate the influence of deformation on network evolution processes in gluten starch matrices and wheat flour doughs in-situ.

By implementing the above-mentioned combination of rheometer and CLSM the rheological and microscopic comparability with classical z-kneaded dough was shown and proved the comparability of the micro system with classical analysis. For the developing dough matrix, an increase in interconnected gluten network at the microscopic level (TNoJs) and network connectivity z at the rheological level was shown and could be directly linked. For the evolution of the continuous gluten phase, the applied shear forces were sufficient to develop the dough matrix over the whole sample cross section. In agreement with

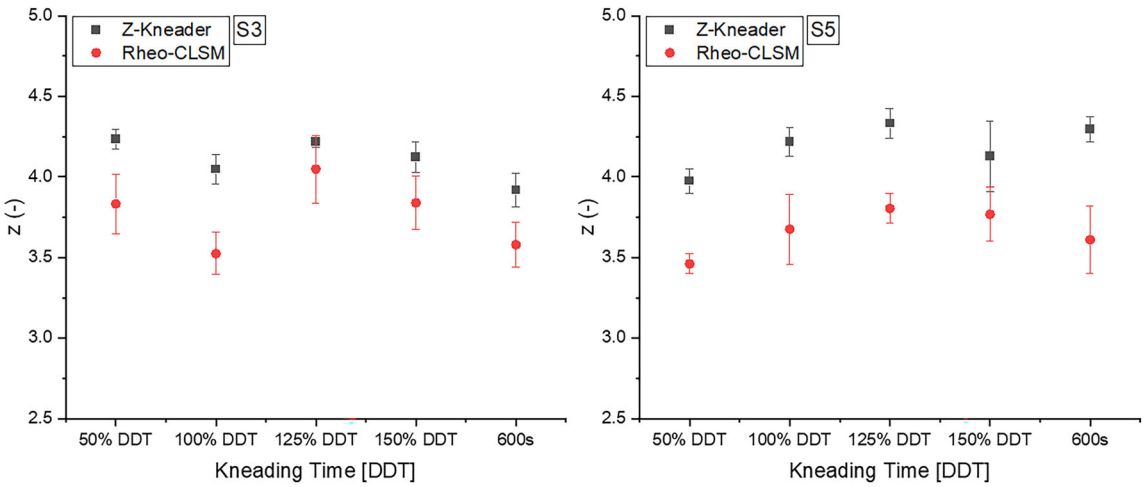

FIGURE 6 Network connectivity z with standard deviations resulting from power-law fitting of the complex modulus measured via frequency sweep with 0.1–50 Hz at 0.05% deformation for wheat flour samples S3 and S5 ($n = 3$).

classically kneaded wheat flour doughs, an optimum development stage was observed which was close to the externally determined stage in a DoughLab. As a result of investigating flours with different a composition, the influence of protein composition on the achievable network attributes (TNoJ, connectivity *z*) was also shown. With a Pearson coefficient of .94 ($p < .05$), the HMW-GS had the greatest impact on the achievable TNoJs within the network. For the rheological units correlated with one another as described by *z*, the LMW-GS showed strong correlations with a Pearson coefficient of .89 ($p < .05$). Since the results were obtained only for native wheat flour, the applicability of the combined shear-kneading-CLSM system must be further investigated for adapting the kneading technique to other gluten-containing samples. The developed method represents a new step toward a fully comprehensible investigation of dough and gluten network development without sample transfer and process interruption for microscopic and rheological analysis.

AUTHOR CONTRIBUTIONS

Leonhard Maria Vidal: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; formal analysis; project administration. **Hans Ewigmann**: Investigation; methodology. **Clemens Schuster**: Investigation. **Thekla Alpers**: Conceptualization; writing – review & editing; project administration. **Katharina Anne Scherf**: Writing – review & editing. **Mario Jekle**: Writing – review & editing; project administration; conceptualization. **Thomas Becker**: Writing – review & editing; supervision; project administration; funding acquisition.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Leonhard Maria Vidal https://orcid.org/0000-0002-8622-5264
Mario Jekle https://orcid.org/0000-0002-2560-0040

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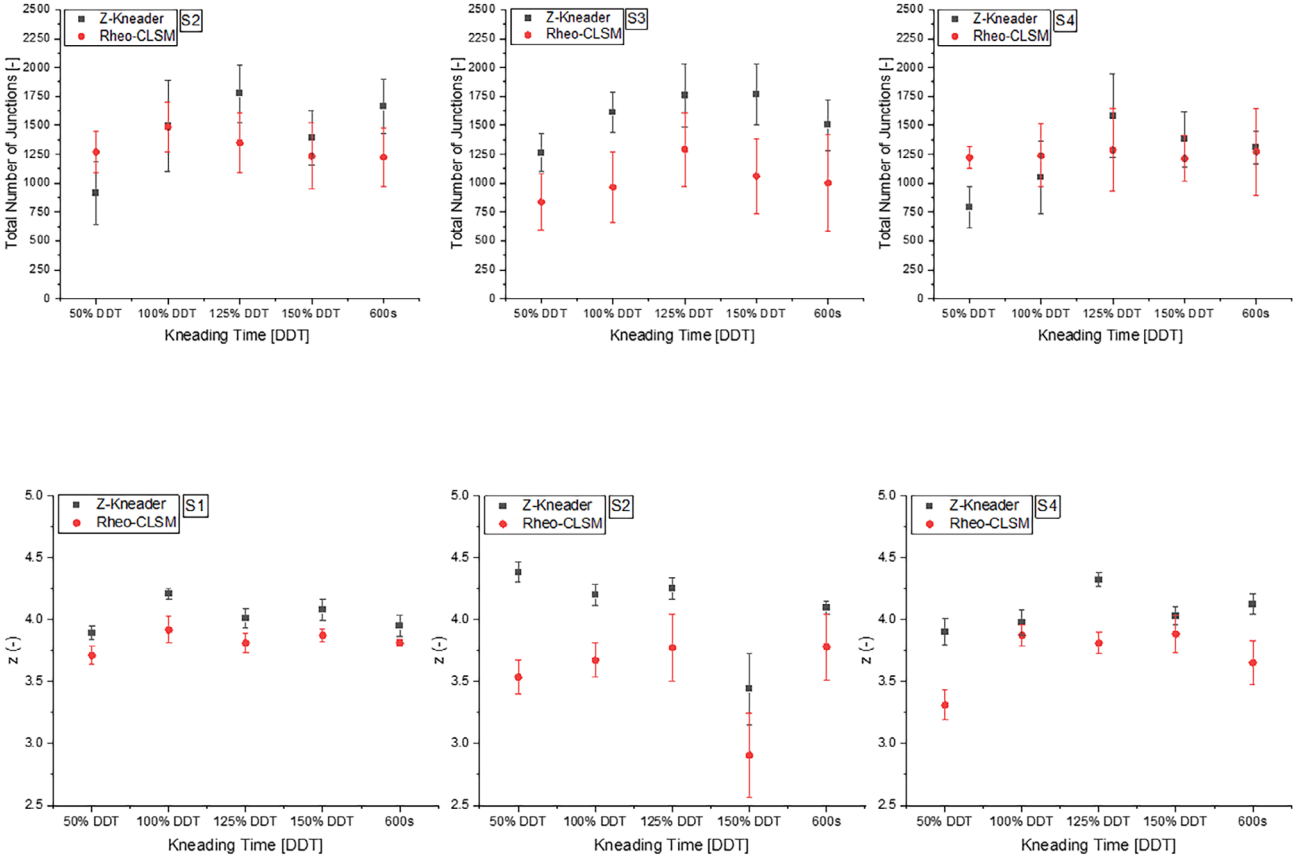
APPENDIX


Z-Kneader
Rheo-CLSM
S2
S3
S4
S1
Total Number of Junctions [-]
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Kneading Time [DDT]
50% DDT
100% DDT
125% DDT
150% DDT
600s