

Rheological Behavior of Food Emulsions Mixed with Saliva: Effect of Oil Content, Salivary Protein Content, and Saliva Type

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Abstract In this paper, we studied the effect of saliva on the rheological properties of β -lactoglobulin- and lysozyme-stabilized emulsions, prepared at pH=6.7 in relation to variation of emulsions- and saliva-related parameters. The effect of oil–volume fraction (2.5% w/w to 10% w/w), salivary protein concentration (0.1 to 0.8 mg ml⁻¹), and the use of both stimulated and unstimulated saliva was investigated. Viscosity and storage modulus were measured before (η_{emul} and G'_{emul} , respectively) and after addition of saliva (η_{mix} and G'_{mix}). To better estimate the changes due to saliva-induced flocculation of the emulsions, the ratios η_{mix}/η_{emul} , G'_{mix}/G'_{emul} were calculated. In addition, $\tan \delta$

(=the ratio of the loss and storage moduli) was investigated to evaluate the viscoelastic behavior of the emulsion/saliva mixtures. Increasing the oil–volume fraction and salivary protein concentration resulted in an increase in η_{mix}/η_{emul} and G'_{mix}/G'_{emul} , while a decrease in $\tan \delta$ of the emulsion/saliva mixtures is occurring. When compared with unstimulated saliva, mixing β -lactoglobulin-stabilized emulsions with stimulated saliva led to a reduction in η_{mix}/η_{emul} and G'_{mix}/G'_{emul} , and an augment of $\tan \delta$ at all measured deformations. In case of lysozyme-stabilized emulsions, the use of stimulated saliva increased G'_{mix}/G'_{emul} for $\gamma < 3$ when compared to unstimulated saliva. The effect of stimulated saliva on the η_{mix}/η_{emul} and $\tan \delta$ in this mixture is similar to that of unstimulated saliva. These results indicate that the influence of stimulated saliva on the rheological parameters of emulsion/saliva mixtures largely depends on the type of emulsions. To conclude, our findings demonstrate that the rheological behavior of emulsions upon mixing with saliva is greatly affected by both saliva and emulsion properties.

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Introduction

Human saliva is involved in several functions such as maintaining oral health, protection of the teeth and mucosal surfaces, and eating^{1–4}. Proteomics revealed that saliva contains more than 1,050 different proteins and peptides⁵ with molecular mass varying from a few kilodaltons to >1,000 kDa, e.g., for the large secreted polymeric mucin MUC5B⁶. Mucins, which are the main constituents of the mucous secretion throughout the body, are a family of

highly glycosylated molecules, carrying at physiological pH a net negative charge due to the presence of sialic acid residues and sulfate groups^{7,8}. Several levels of protein organization, e.g., the presence of the network composed of MUC5B and the so-called “salivary micelles”^{9–11}, add to the structural complexity of saliva^{12–14} and influence its rheological properties. In particular, the type of mucin and its origin appear to play a dominant role in the viscoelastic properties of saliva¹⁵. Saliva is secreted by different glands and is susceptible to variation depending on many factors as type of the salivary gland or stimuli^{16,17}. The exact amount of this oral fluid in the oral cavity is not known as a result of a dynamic process involving both constant saliva production and swallowing. However, in resting conditions, it is generally assumed to be between 1 and 1.5 ml. Unstimulated saliva is mainly secreted from sublingual and submandibular glands, while the parotid gland contributes for about 80% of the total stimulated saliva production. Parotid saliva does not contain mucins and has a shear-rate-independent viscosity slightly higher than water^{15,18}. Sublingual saliva, instead, shows a clear shear-thinning behavior¹⁵ and plays an important role in the prevention of oral dryness because of its high viscosity and elasticity. Submandibular saliva exhibits lower elasticity than sublingual saliva. This characteristic is important for the lubrication during speaking and swallowing, and for bolus formation¹⁵. The consumer’s perception of food products is becoming increasingly important for the food industry in relation to product design and evaluation. Attempts have been made to correlate sensory perceived attributes with physical parameters of the products, as for example, the in-mouth thickness (perceived thickness) of fluids and semisolid foods with the shear viscosity¹⁹ or the shear stress^{20,21}. Moreover, several authors reported the influence of saliva properties, such as flow, composition, and lubrication, on sensory perception and flavor release^{22–25}. It is becoming evident that knowledge on the interaction of food products with saliva is important for understanding oral processing of food because, often, perception cannot be directly related to the texture of the products before consumption. For example, the correlation between the shear-thinning behavior of different polysaccharide solutions and mouth-feel sliminess, which was formulated over 30 years ago²⁶, is not clearly established, as no correlation was observed in subsequent investigations²⁷. Therefore, investigations of the dynamic processes occurring in the mouth, which are affecting the food structure, were initiated. In our research, we focused on liquid food emulsions stabilized by proteins that undergo flocculation after mixing with the saliva^{28,29}. A clear role of droplet charge on the emulsion stability and viscosity upon mixing with saliva was established by using differently charged surfactants and proteins as emulsifiers³⁰. Strongly nega-

tively charged emulsions did not flocculate in the presence of saliva, whereas weakly negatively charged emulsion droplets, stabilized by β -lactoglobulin, reversibly flocculated with respect to dilution and shear. Saliva-induced flocculation of positively charged emulsions, e.g., stabilized by lysozyme, was instead irreversible upon dilution and shear. In particular, for lysozyme, complex formation between lysozyme-stabilized emulsion droplets and salivary proteins was demonstrated³¹.

Rheological measurements are widely used to evaluate emulsion behavior under different applied conditions^{32–34}, to assess long-term stability³⁵, or to characterize flocculation³⁶. Several parameters, such as oil–volume fraction (ϕ) and characteristics of the continuous phase, contribute to the rheological properties of emulsions. It is known that if the effects of colloidal interparticle interactions are negligible, emulsions with $\phi < 0.5$ behave Newtonian³⁷. In case of flocculation, rheology depends on the type and strength of the interaction between the droplets. Flocculated emulsions exhibit a higher viscosity (η), as a result of the increase in the effective volume fraction, and a shear-thinning behavior³⁸. Storage (G') and loss moduli (G'') of these emulsions are usually also evaluated by means of oscillatory measurements^{32,34–36,38}. Flocculation is frequently accompanied by a rapid increase in the storage modulus³⁵ with irreversibly flocculated emulsions showing larger storage moduli than reversibly flocculated emulsions³⁴.

In this paper, we studied the influence of different parameters on the rheological properties, i.e., η , G' , and $\tan \delta$ (i.e., G''/G') of a negatively charged emulsion stabilized by β -lactoglobulin and a positively charged emulsion stabilized by lysozyme after mixing with saliva. The aim of this work was to illustrate how oil–volume fraction, type of saliva, and salivary protein content influence flocculation behavior and, consequently, the rheological properties of emulsion/saliva mixtures.

Materials and Methods

Materials

Freeze-dried β -lactoglobulin (β -lg) was provided by Wageningen Centre for Food Science (WCFS, Wageningen, The Netherlands) and was purified as described previously³⁹. The powder contains 93.6% w/w proteins ($N \times 6.38$). Lysozyme from chicken egg white (L6876 batch 051K7028) was obtained from Sigma-Aldrich Chemie B. V. (Zwijndrecht, The Netherlands) and used without further purification. Lysozyme from chicken egg white from the batch number 016K1189 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was instead desalted on a

Sephadex G10 (flow=40 ml min⁻¹ and pressure=0.4 mPa, eluted with water de-gassed with He). The eluent was freeze dried and stored at -20°C until further use. Without this treatment, lysozyme solutions obtained from this batch number had an acid pH and could not be used to make stable emulsions according to the method indicated in the following paragraph. Sunflower oil (Reddy, Vandemoortele, The Netherlands) was purchased from a local retailer; BCA™ Protein Assay Kit from Pierce Biotechnology Inc. (Rockford, IL, USA), and sodium azide was obtained from Merck (Shuchardt, Germany).

Collection and Handling of Saliva

Unstimulated Saliva Whole human unstimulated saliva was collected according to the previously described procedure³⁰. Briefly, whole human unstimulated saliva was collected from 8:30 to 10:30 A.M. from ten healthy non-medicated volunteers. After rinsing their mouths with water, saliva was collected with closed lips for a couple of minutes and then expectorated into ice-chilled vessels. The first milliliter of saliva was discarded.

Stimulated Saliva Whole human stimulated saliva was collected from the same group of volunteers from 8:30 to 10:30 A.M. In line with our protocol for unstimulated saliva donation, after optionally having breakfast and brushing their teeth, donors refrained from eating and drinking, with the exception of water, for 2 h before donation. After rinsing their mouths with water, the volunteers chewed a piece of parafilm of 5×5 cm in dimension for 6 min. During this time, stimulated saliva was expectorated into ice-chilled vessels every 30 s. Also in this case, the first milliliter of saliva was discarded.

Handling During collection and handling, the samples were constantly kept on ice. Both unstimulated and stimulated saliva were separately pooled and centrifuged at 10,000×g for 30 min at 4°C to remove cellular debris (Beckman, model Avanti™ J-25 I, rotor JA-21, Beckman Coulter B.V. Mijdrecht, The Netherlands). The supernatants were frozen in liquid nitrogen, stored at -80°C and used within 6 weeks. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry experiments conducted to test the effect of different treatments and storage conditions on the low-molecular-weight salivary proteins have shown that the used handling conditions have little effect on saliva when compared with fresh saliva⁴⁰. No precipitation of salivary proteins was observed after thawing. The pH of unstimulated saliva ranged from 6.7 to 7.0, while the pH of stimulated saliva was about 8. The content of salivary proteins, indicated as SPs, both of stimulated and unstimulated saliva, was determined according to the BCA method

of Pierce using bovine serum albumin as standard. The SPs varied from 1.1 to 1.3 mg ml⁻¹ for unstimulated saliva and about 0.7 mg ml⁻¹ for stimulated saliva. In this paper, with the term saliva, we indicate pooled unstimulated saliva after the handling procedure.

Preparation and Characterization of O/W Emulsions

β-Lactoglobulin- and lysozyme solutions were prepared by dissolving the protein powder (1% w/w) overnight at 4°C in demineralized water and 10 mM NaCl solution (59 g), respectively. Pre-emulsions were prepared using an Ultra-Turrax T 25 Basic (IKA-Werke GmbH & Co. KG, Staufen, Germany), and subsequently, stock emulsions were homogenized at room temperature by ten passes through a Delta Lab-scale homogenizer (Delta Instruments B.V., Drachten, The Netherlands). The operating pressure was 70 bars for β-Ig stabilized emulsions and 100 bars in case of lysozyme emulsions. β-Ig stabilized stock emulsions made at pH 6.7 contained 40% w/w sunflower oil and 1% w/w emulsifier, while lysozyme-stabilized stock emulsions (pH 6.7, 1% w/w protein and 10 mM NaCl) contained 20% w/w sunflower oil. Sodium azide (0.02% w/w) was added to the emulsions to prevent microbial growth.

Light microscopy images were taken using an Olympus BX 60 Microscope equipped with an Olympus DP 70 camera (Olympus Nederland B.V., Zoeterwoude, The Netherlands). Droplet-size distribution and the volume-over surface average droplets diameter (d_{32}) were measured by laser diffraction with the Mastersizer Hydro 2000S (Malvern Instruments, Southborough, UK) as described before³⁰.

Sample Preparation for Rheology Experiments

Emulsion/saliva mixtures were prepared at room temperature by adding thawed saliva to diluted β-Ig- and lysozyme-stabilized emulsions containing 10 mM NaCl in the bulk phase. To reduce proteolytic activity, which is naturally present in saliva, samples were thawed at room temperature shortly before each experiment. The effect of oil-volume fraction (ϕ) was studied by varying the oil phase between 2.5% w/w and 10% w/w in emulsion/saliva mixtures containing 0.6 mg ml⁻¹ SPs, in line with a previous paper where emulsions were mixed 1:1 with saliva²⁸. The influence of SPs on the emulsion/saliva mixture has been determined at two different oil contents (2.5% w/w and 10% w/w). SPs was therefore varied between 0.1 and 0.6 mg ml⁻¹ in a mixture containing a 10% w/w oil phase and between 0.1 and 0.8 mg ml⁻¹ in a mixture containing 2.5% w/w oil phase. Lastly, the effect of saliva type (stimulated vs. unstimulated) was determined in a mixture containing 0.4 mg ml⁻¹ SPs and 10% w/w oil. Moreover,

the rheological properties of emulsions alone at different ϕ were used as references and were measured on β -lg- and lysozyme-stabilized emulsions diluted to the same volume fraction of emulsion/saliva mixture by addition of 10 mM NaCl solution.

Rheology

The shear-rate-dependent viscosity of emulsions, saliva, and the mixtures was measured in duplicate, using a Physica MCR 301 rheometer (Anton Paar BVBA, Sint Martens Latem, Belgium) at 20°C, using a cone-and-plate geometry CP 75-1 with an angle of 1° (0.0175 rad) and a gap-width of 0.05 mm at the tip. The shear rate was logarithmically increased over 20 min from 0.1 to 1,500 s⁻¹. Viscoelasticity measurements were carried out on emulsions, saliva, and their mixtures using the Vilastic-3 viscoelasticity analyzer (Vilastic Scientific Inc., Austin, Texas, USA). This instrument is mostly used in measurements of low-viscosity fluids, in particular blood^{41–43} but also saliva¹⁵. Measurements were performed in duplicate using oscillatory flow in a vertical capillary cylinder with the dimension of 63.97 mm in length and 5.04 mm in diameter. The pressure drop and volume flow across the tube are measured with an accuracy of 2%. The pressure and flow are related to the shear stress, shear strain, shear rate, and viscoelasticity of the fluid as described by Thurston for the oscillation of a viscoelastic fluid in a circular tube^{44,45}. According to the manufacturer's protocol, a frequency of 2 Hz was selected. The storage and loss moduli were consequently measured in the shear strain (γ) range between 0.002 and 19, and shear viscosity in the shear rate range between 0.01 and 250 s⁻¹. The results from the viscoelasticity analyzer were in line with those obtained with the cone-and-plate geometry measurements but covered a smaller shear rate range. For this reason, in case of

shear viscosity, we show only the findings from the Physica MCR 301 rheometer measurements.

As a tool to determine the increased viscosity due to saliva-induced flocculation independently from the emulsion viscosity under experimental conditions, we calculated the ratio $\eta_{\text{mix}}/\eta_{\text{emul}}$, where the viscosity of the mixtures (η_{mix}) is normalized by the viscosity of the emulsions (η_{emul})³⁰. To evaluate saliva influence on viscoelastic parameters, we determined the ratio $G'_{\text{mix}}/G'_{\text{emul}}$, where G'_{mix} and G'_{emul} are the storage modulus of the mixtures and emulsions before mixing with saliva, respectively. In addition, $\tan \delta$, defined as the ratio between the loss modulus and the storage modulus (G''/G'), is shown as well. As the observed trends were independent of the applied strain, we chose to present the results at a deformation of 1.1.

Results

Emulsion and Saliva Characterization

Microscopic images of the prepared emulsions revealed that emulsion droplets were homogeneously dispersed throughout the sample and emulsion flocculation was not observed (not shown). Table 1 summarizes the d_{32} and the related measured rheological parameters, i.e., η , G' , and $\tan \delta$ of the prepared emulsions at different oil contents. d_{32} were similar for β -lg- and lysozyme-stabilized emulsions. In line with previous measurements³⁰, both emulsions exhibited Newtonian behavior and displayed similar viscosity values (Table 1). G' and $\tan \delta$ were strain-independent at all studied oil contents with a small but detectable increase in η and G' upon increasing the oil content (2.5% w/w vs. 10% w/w).

Rheological parameters of unstimulated and stimulated saliva are also reported in Table 1. Viscosities of saliva (1.15 mPa.s for stimulated and 1.18 mPa.s for unstimulated

Table 1 d_{32} , viscosity (η), storage modulus (G') and $\tan \delta$ of β -lactoglobulin- and lysozyme-stabilized emulsions at different oil content (% w/w)

| Sample | Oil (% w/w) | d_{32} (μm) | η (mPas) ^a | η (mPas) ^b | G' (mPa) ^b | $\tan \delta$ ^b |
|------------------------|-------------|----------------------------|----------------------------|----------------------------|-------------------------|----------------------------|
| β -Lactoglobulin | 2.5 | 0.98 (0.01) | 1.11 (0.01) | 1.10 (<0.01) | 1.17 (0.13) | 12.05 (0.20) |
| | 5 | 1.10 (0.01) | 1.34 (0.01) | 1.18 (<0.01) | 1.18 (0.13) | 12.50 (0.11) |
| | 10 | 1.12 (0.01) | 1.43 (<0.01) | 1.44 (<0.01) | 1.55 (0.16) | 11.03 (0.05) |
| Lysozyme | 2.5 | 1.14 (0.07) | 1.07 (0.06) | 1.07 (<0.01) | 1.50 (0.02) | 9.00 (0.10) |
| | 5 | 0.98 (0.03) | 1.28 (0.17) | 1.28 (0.19) | 1.53 (0.05) | 11.64 (5.00) |
| | 10 | 1.10 (0.08) | 1.39 (0.03) | 1.27 (<0.01) | 1.74 (0.07) | 9.21 (0.09) |
| Unstimulated saliva | – | – | 1.18 (0.32) | 1.23 (<0.01) | 1.94 (0.17) | 7.74 (0.18) |
| Stimulated saliva | – | – | 1.15 (0.01) | 1.16 (0.01) | 1.34 (0.07) | 11.60 (0.04) |

η , G' , and $\tan \delta$ of unstimulated and stimulated saliva for a SPs concentration of 0.6 mg ml⁻¹ is reported as well. Standard deviation is shown in parenthesis

^a Values measured with cone-and-plate geometry at 100 s⁻¹

^b Values measured with capillary setup at 95 s⁻¹ and $\gamma=1.1$

saliva) are in line with the values reported in literature^{15,46}. G' and $\tan \delta$ were strain-independent for both unstimulated and stimulated saliva. In line with the known weak gel-like character attributed to unstimulated saliva, G' is slightly higher than that in stimulated saliva, while $\tan \delta$ showed an opposite behavior.

Effect of Oil Content

The effect of oil content on the rheological properties of β -lg- and lysozyme-stabilized emulsions after mixing with saliva was analyzed as a function of the shear rate and deformation. As the two emulsions showed a similar trend in saliva-induced viscosity changes ($\eta_{\text{mix}}/\eta_{\text{emul}}$), we report our findings only for β -lg emulsion/saliva mixtures (Figure 1). Increasing the oil content led to an increase of $\eta_{\text{mix}}/\eta_{\text{emul}}$ at all applied shear rates. Although not easily visible, small differences in $\eta_{\text{mix}}/\eta_{\text{emul}}$ are still present at high shear rate ($>560 \text{ s}^{-1}$).

The influence of the ϕ on $G'_{\text{mix}}/G'_{\text{emul}}$ and $\tan \delta$ is exemplified in Figure 2 for both emulsions. Increasing the oil content led to an augment of $G'_{\text{mix}}/G'_{\text{emul}}$. Large differences in the ratios between the mixtures of saliva with the two emulsions have been seen, with lysozyme-stabilized emulsion/saliva mixture showing, at 10% w/w, a threefold higher value of $G'_{\text{mix}}/G'_{\text{emul}}$ than the β -lg emulsion/saliva mixture. By lowering the oil content, the difference between the two mixtures became smaller until it disappeared at 2.5% w/w oil content. Opposite to storage modulus and in line with the expectations, $\tan \delta$ decreased upon increasing the oil content for both emulsion/saliva mixtures, indicating that saliva increases the

elastic component of the mixtures as ϕ becomes larger (Figure 2).

Influence of Salivary Protein Content and Type of Saliva

Figure 3 summarizes the effect of SPs on the saliva-induced viscosity increase for β -lg- and lysozyme-stabilized emulsions at two different oil contents (2.5% w/w and 10% w/w). Typically, increasing the SPs concentration in the mixtures resulted in an augment of the ratio $\eta_{\text{mix}}/\eta_{\text{emul}}$. This effect is most pronounced at 10% w/w for both emulsion types. In addition, in line with previous results³⁰, the irreversibly flocculated lysozyme-stabilized emulsion/saliva mixtures show a larger viscosity increase compared to the reversibly flocculated β -lg-stabilized emulsion/saliva mixtures prepared at the same ϕ . It is worth to note that comparable viscosity ratios could be obtained, for example, when 0.4 mg ml^{-1} SPs was added to a β -lg-stabilized emulsion (10% w/w) and a lysozyme-stabilized emulsion (2.5% w/w). Clearly a combination of both SPs concentration and ϕ is affecting the saliva-induced flocculation behavior of emulsions and therewith the ratio $\eta_{\text{mix}}/\eta_{\text{emul}}$.

To conclude the analysis of the influence of SPs concentration on the rheological parameters of emulsion/saliva mixtures, we illustrate in Figure 4 the $G'_{\text{mix}}/G'_{\text{emul}}$ and $\tan \delta$ values for β -lg-stabilized emulsion/saliva mixtures at 10% w/w oil. An increase in $G'_{\text{mix}}/G'_{\text{emul}}$ and a reduction in $\tan \delta$ as function of the SPs concentration are observed. Similar behavior has been observed for the other studied oil–volume fractions, as well as for lysozyme-stabilized emulsion/saliva mixtures.

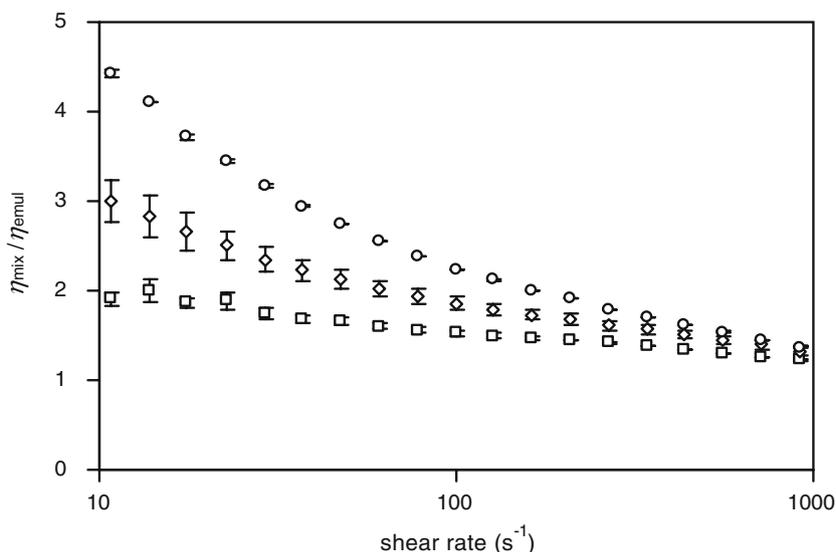


Fig. 1 Effect of oil content on the shear-rate-dependent $\eta_{\text{mix}}/\eta_{\text{emul}}$ for β -lg-stabilized emulsions after mixing with saliva (0.6 mg ml^{-1} SPs) at 2.5% w/w (square), 5% w/w (diamond), and 10% w/w (circle) oil phase. Error bars represent the standard deviation

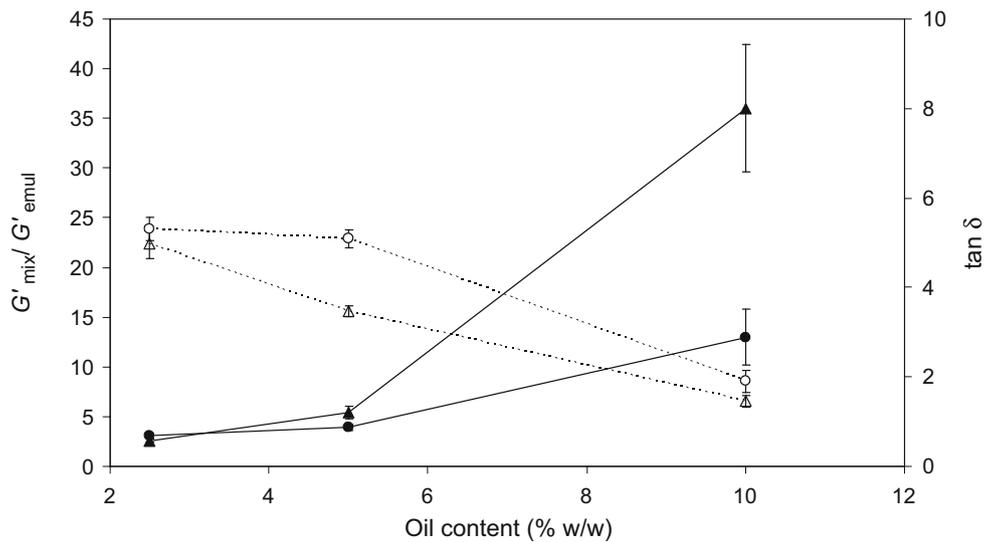


Fig. 2 Effect of oil content on the G'_{mix}/G'_{emul} (closed symbols) and $\tan \delta$ (open symbols) measured at $\gamma=1.1$ for β -lg- (filled/open circle) and lysozyme (filled/open triangle)-stabilized emulsions after mixing

with saliva (0.6 mg ml⁻¹ SPs). Error bars represent the standard deviation. Lines are intended to guide the eye

The different effect of unstimulated and stimulated saliva on the shear-rate-dependent η_{mix}/η_{emul} of β -lg- and lysozyme-stabilized emulsions is shown in Figure 5. As the protein concentration in stimulated saliva was lower than in unstimulated saliva, we prepared emulsion/saliva mixtures at 10% w/w and 0.4 mg ml⁻¹ instead of the usually used concentration of 0.6 mg ml⁻¹. The type of saliva had a major effect on β -lg-stabilized emulsions because, as shown, stimulated saliva induced a lower ratio η_{mix}/η_{emul} compared

to unstimulated saliva at shear rates below 300 s⁻¹. A minor effect of the type of saliva on η_{mix}/η_{emul} has been seen for lysozyme-stabilized emulsion/saliva mixtures.

In line with the effect on the viscosity (Figure 5), the use of stimulated saliva in β -lg-stabilized emulsion/saliva mixtures resulted in smaller G'_{mix}/G'_{emul} compared to unstimulated saliva (Figure 6a). Moreover, it is noted that G'_{mix} , when stimulated saliva was used, was lower compared to G'_{emul} , which resulted in the illustrated $G'_{mix}/G'_{emul} < 1$ (Figure 6a).

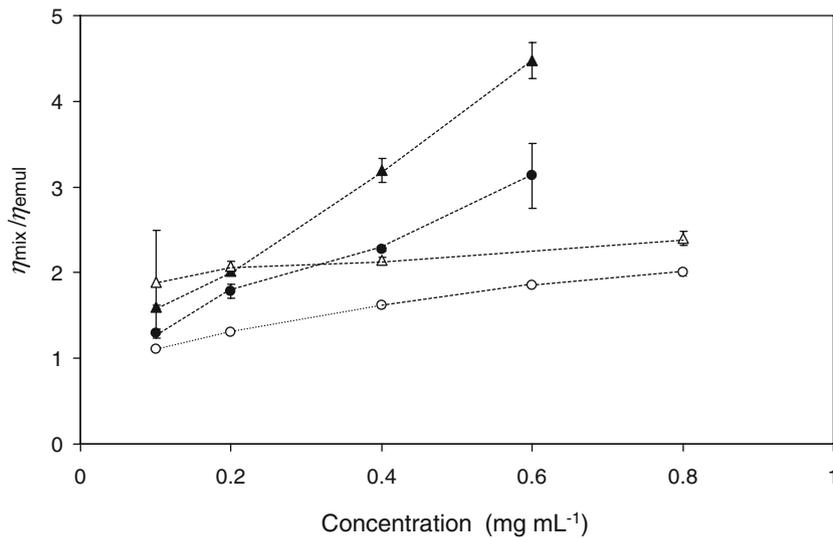


Fig. 3 Effect of salivary protein concentration on η_{mix}/η_{emul} at 100 s⁻¹ for β -lg-stabilized emulsions (filled/open circle) and lysozyme stabilized emulsions (filled/open triangle) after mixing with saliva at

2.5% w/w oil content (open symbols) and 10% w/w oil content (closed symbols). Error bars represent the standard deviation. Lines are intended to guide the eye

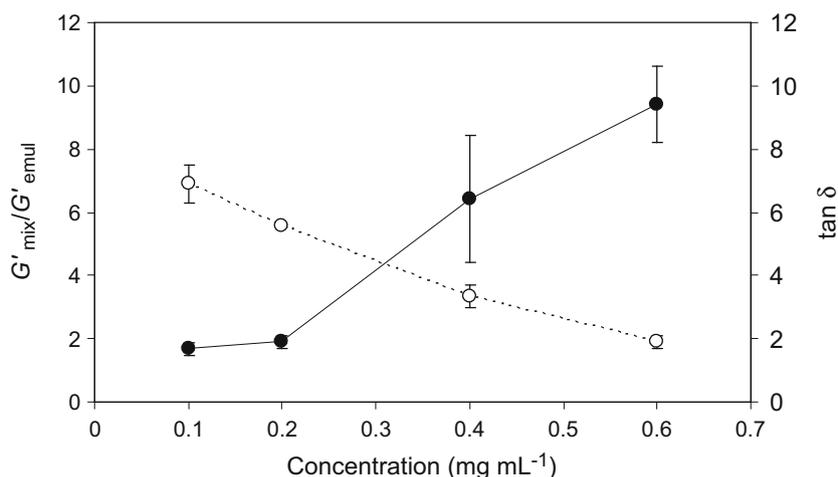


Fig. 4 Effect of SPs concentration on the strain-dependent G'_{mix}/G'_{emul} (closed symbols) and $\tan \delta$ (open symbols) for β -lg-stabilized emulsions (10% w/w) after mixing with saliva at $\gamma=1.1$. Error bars represent the standard deviation. Lines are intended to guide the eye

As the G'_{mix} values were obtained in the measurable range of the instrument provided by the manufacturer, we cannot provide a plausible explanation for this result. Figure 6a illustrates the $\tan \delta$ measured in case of both unstimulated and stimulated saliva. As shown, $\tan \delta$ is higher at all the measured strains when stimulated saliva was used. Analogously, Figure 6b reports G'_{mix}/G'_{emul} and $\tan \delta$ for lysozyme-stabilized emulsions after addition of saliva. Remarkably, stimulated saliva induced higher G'_{mix}/G'_{emul} than unstimulated saliva for $\gamma < 3$, while no substantial differences were observed in the $\tan \delta$ when two saliva types were used.

Discussion

In this paper, we studied the effect of different parameters on saliva-induced flocculation of emulsions stabilized by β -lactoglobulin (pI~4.9) and by lysozyme (pI~10.5) at neutral pH. As we have previously reported, emulsions flocculated upon the addition of saliva^{28,30}, which enhanced the viscosity and storage modulus, and decreased the $\tan \delta$. The changes in these parameters induced by saliva, presented as the ratios η_{mix}/η_{emul} , G'_{mix}/G'_{emul} , can be of relevant for understanding the oral perception of liquid emulsions. Identification of measurable physical properties

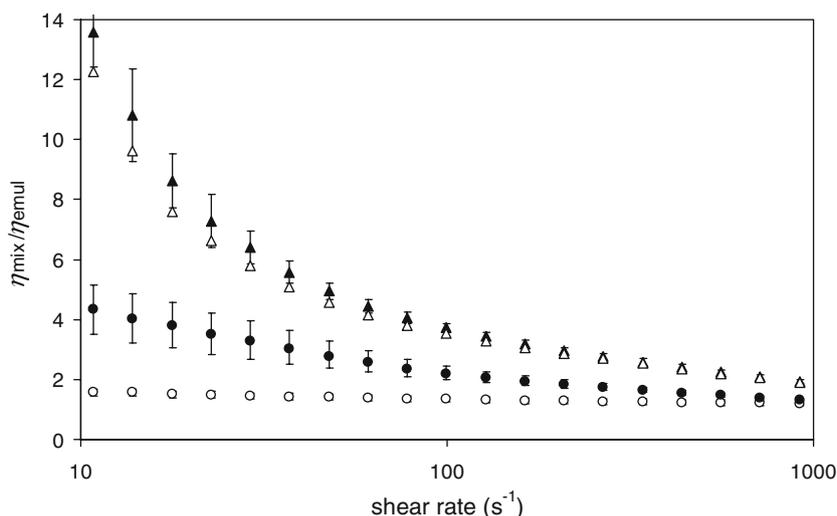


Fig. 5 Effect of stimulated (open symbols) and unstimulated (closed symbols) saliva on the shear-rate-dependent η_{mix}/η_{emul} for β -lg- (filled/open circle) and lysozyme-stabilized emulsions (filled/open triangle)

after mixing with saliva (10% w/w oil phase; 0.4 mg ml⁻¹ SPs). Error bars represent the standard deviation

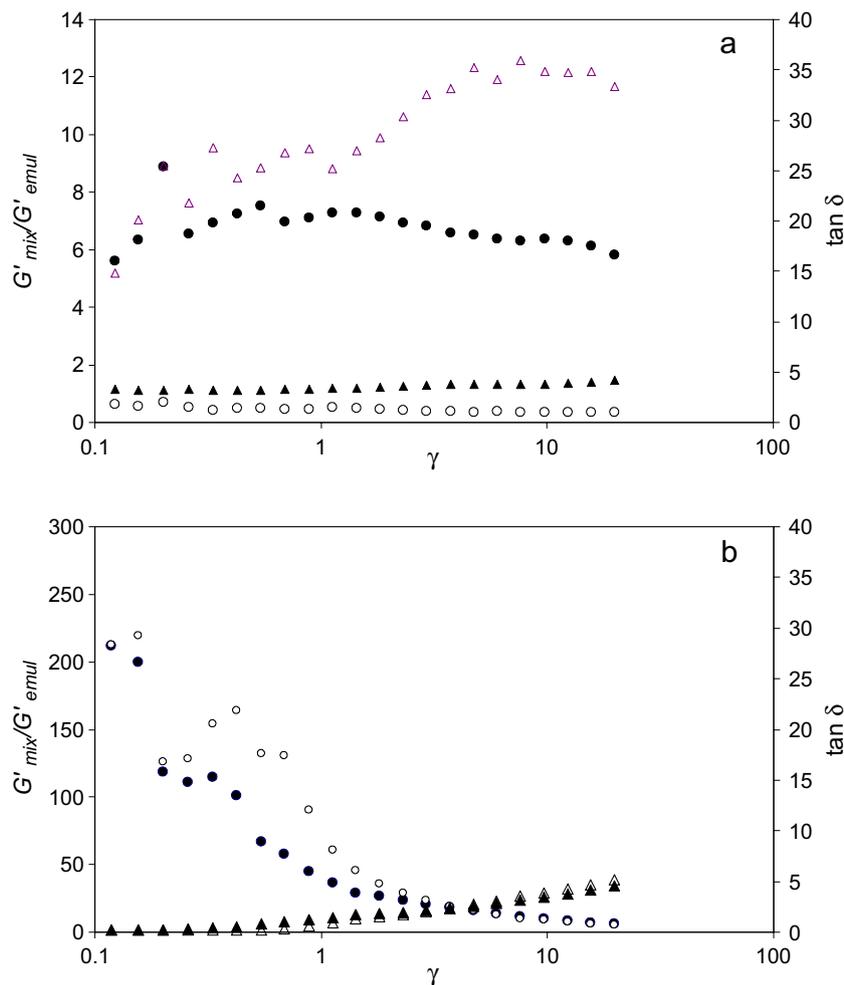


Fig. 6 Effect of stimulated (*open symbols*) and unstimulated (*closed symbols*) saliva on G'_{mix}/G'_{emul} (filled/open circle) and $\tan \delta$ (filled/open triangle) for β -lg-stabilized emulsions (Figure 6a) and lyso-

zyme-stabilized emulsions (Figure 6b) after mixing with saliva (10% w/w oil phase; 0.4 mg ml⁻¹ SPs)

to predict perceived texture and mouth-feel has been the focus of several studies. A number of attempts has been made to establish correlations between sensory and rheological properties as well as to determine the exact conditions, e.g., shear stress and shear rate, in the mouth^{19–21,47}. It is generally accepted that viscosity enhancement plays an important role in oral sensory perception of fluid and semi-solid foods^{48,49}. Our study shows that saliva generally affects the viscoelasticity of the emulsions upon mixing by mainly changing the elastic component of the mixture, i.e., increase in G'_{mix}/G'_{emul} and decrease of $\tan \delta$. Correlations between viscosity, storage modulus, and $\tan \delta$ with sensory perception of emulsions are beyond the scope of this article. Nevertheless, our results indicate the importance of including the contribution of saliva to emulsion rheology when attempting to clarify the relation between emulsion properties and sensory perception.

One of the main factors affecting emulsion rheology is the volume fraction of the dispersed phase. Several studies demonstrated the effect of volume fraction by illustrating the relationship between the emulsion relative viscosity and ϕ (up to 0.6–0.8) or by reporting the influence of ϕ on the stability of a flocculated emulsion^{50–52}. In non-flocculating diluted emulsions, the viscosity can be theoretically calculated by using, for example, the Batchelor equation⁵³, where the viscosity of the emulsion depends on the viscosity of the continuous phase and the oil–volume fraction of the droplets (up to $\phi=0.2$)³⁶. In flocculated emulsions, as obtained by the addition of salivary components, the volume fraction of the flocculated droplets is higher than ϕ , as the continuous phase is also included in the floc structure. These emulsions exhibit a higher viscosity and a shear-thinning behavior^{28,51,54}. As expected from literature, raising the oil content up to 10% w/w increases the ratios η_{mix}/η_{emul} and G'_{mix}/G'_{emul} , and reduces $\tan \delta$ as a

consequence of the enhanced saliva-induced flocculation occurring in the system.

The addition of polymers to the continuous phase and its effect on the stability of emulsions is extensively discussed in the emulsion literature in relation to creaming^{51,54–56}, flocculation^{57–59}, and rheological characteristics^{32,60}. By modulating the concentration of added polymer, which induces changes in the internal structure of the emulsions, the interaction between the droplets can be controlled. Although, in some cases, the experiments have been carried out at different emulsion volume fractions, it has been generally observed that viscosity, shear stress, and storage modulus increase with increasing polymer concentration^{32,60,61}.

In view of its composition, saliva can be considered as a colloidal dispersion of highly structured biopolymers, e.g., salivary mucins, and micelles. Secreted salivary mucins, MUC5B and MUC7, can be considered as negatively charged polysaccharides because, as reported by Zalewska⁶², 40–80% of the mass of such mucins consist of O-linked oligosaccharides. Sialic acid and sulfate groups, as well as aspartic and glutamic acids, provide the molecule with a negative charge⁶². The effect of salivary protein content was investigated in emulsion/saliva mixtures at 2.5% *w/w* and 10% *w/w* oil content. Similar to the oil content observations and in line with results reported in literature for other polysaccharides, raising the SPs concentration increased $\eta_{\text{mix}}/\eta_{\text{emul}}$ and $G'_{\text{mix}}/G'_{\text{emul}}$, and reduced $\tan \delta$. In view of the fact that a liquid emulsion remains in the mouth generally less than 10 s before being swallowed, it is likely that the emulsions are mixed with saliva in a heterogeneous way. Therefore *in vivo*, the physical–chemical features of a mixture may depend on the relative amounts of both saliva and oil, and may vary with the oral movements and the proximity of the emulsions to salivary glands. This may cause inhomogeneous mixing of the sample. Therefore, we anticipate that the combination of both these factors, i.e., salivary protein concentration as well as oil content, might be of extreme relevance for understanding the real behavior of an emulsion in the mouth.

As saliva is secreted by different glands and its properties are affected by stimulation, we compared the effect of stimulated saliva, obtained by chewing on a piece of parafilm, with that of unstimulated saliva. Saliva stimulation influences the protein composition, in particular the type of secreted proteins and their concentration^{1,17,63}. Unstimulated saliva is composed of several proteins including α -amylase, serum albumin, immunoglobulin, and mucin, which make up for 20–30% of the protein content⁶⁴. Stimulated saliva contains, among others, α -amylase, proline-rich protein, and a lower amount of mucin (MUC5B and MUC7). MUC7 was found in whole stimulated saliva and parotid saliva as one of the components of the salivary micelles, together with lysozyme,

lactoferrin, α -amylase, and glycosylated proline-rich protein⁶⁵. Salivary micelles are globular structures with sizes in the range 40–500 nm^{9,66} with negative surface potential at physiological pH^{66,67}.

As observed in Figure 5, stimulated saliva induced lower viscosity in β -lactoglobulin-stabilized emulsion/saliva mixtures than unstimulated saliva. This is in line with the previous hypothesis²⁸ that mucins were causing the flocculation of β -lg emulsions by depletion. In fact, the concentration of salivary mucins is lower in stimulated saliva than in unstimulated saliva, as up to 80% of saliva during stimulation is secreted by the parotid glands. The observed flocculation of β -lactoglobulin-stabilized emulsions upon mixing with stimulated saliva is likely induced by both salivary mucins and salivary micelles, as previously suggested²⁸.

In case of lysozyme-stabilized emulsions, we showed that complex formation between emulsion and salivary proteins was responsible for the irreversible flocculation of these emulsions³¹. In particular, MUC5B is likely to play a role in this process because pig gastric mucin, which is often used as model for the MUC5B, has shown strong interaction with cationic macromolecules such as gelatins and chitosan⁶⁸. Besides MUC5B, other salivary proteins such as amylase could also interact with the positively charged droplets, explaining therefore the small difference observed when the two different types of saliva were used. The relative amount and type of proteins produced with the secretion of stimulated saliva might strengthen the complex formation between the saliva and the lysozyme-stabilized emulsion droplets, and this could explain the observed behavior of $G'_{\text{mix}}/G'_{\text{emul}}$ and the small effect on the $\eta_{\text{mix}}/\eta_{\text{emul}}$ and $\tan \delta$.

Conclusions

This study aimed to elucidate the effect of several parameters, i.e., oil–volume fraction, salivary protein concentration, and type of saliva, on the rheological properties of saliva-induced flocculated emulsions. We showed that saliva-induced flocculation increased, as expected, the emulsion viscosity and storage modulus while decreased the $\tan \delta$. In particular, a larger increase in the storage modulus was observed in both β -lactoglobulin- and lysozyme-stabilized emulsions upon mixing with unstimulated saliva. Moreover, increasing the oil–volume fraction and the amount of salivary proteins increased the viscosity, storage modulus, and consequently reduced $\tan \delta$. Lastly, the effect of the type of saliva was analyzed. The results indicated that stimulated saliva causes a smaller increase of the viscosity of β -lactoglobulin-stabilized emulsion compared to unstimulated saliva. A decrease in storage modulus and an augment in $\tan \delta$ were observed

upon mixing stimulated saliva with β -lactoglobulin-stabilized emulsions. In case of the lysozyme-stabilized emulsion/saliva mixtures, compared to unstimulated, stimulated saliva influenced the storage modulus but did not significantly affect the viscosity and $\tan \delta$ of this mixture.

With this study, we demonstrated that saliva has a great influence on emulsion properties and that the rheological behavior is determined by both emulsion properties and saliva characteristics. Therefore, we advise to include the contribution of saliva in studies that aim to understand the oral perception of food emulsions.

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