Microbrewidics: a microfluidic platform to investigate what stabilizes hop oil emulsions in beer

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Abstract

Hop oils form microscopic emulsions in aqueous beer, but little is known about which molecules in beer stabilise these emulsions. Here we use a microfluidic platform as a tool to enable the creation of assays to explore the role of proteins in the stabilisation of hop oil emulsions in beer. The terpenes linalool and α-pinene were used to form emulsions with a Kölsch-style ale on a microfluidic device (oil-in-beer emulsions). Gluten was added to these emulsions on-chip to investigate how this protein, which is present in beer, affects the stability of the emulsions. Then Brewers Clarex, an enzyme commonly

used in brewing to degrade proteins, was added to digest the oil-in-beer emulsions. Our data suggest that the type and amount of proteins present in beer may affect the stability of the hop oil emulsions, which could have an impact on the shelf life and sensory quality of the beer.

Introduction

While brewers are well known for experimentation to create flavourful and innovative beers, the precise mechanisms by which they achieve these remain, in some cases, unknown. Beer is a complex mixture of esters, alcohols, organic acids, saccharides, metals, proteins, tannins and many other classes of compounds contributed by raw materials such as malted barley and hops, as well as by-products of yeast fermentation.[1] A detailed understanding of how these ingredients and their by-products affect flavour allows researchers and brewers alike to have more control over both the sensory and visual characteristics of their beers, with the ultimate objective being the ability to maintain the flavour characteristics typical of a fresh beer over many months of shelf life. However, the sheer number of ingredients needed to brew a tasty beer makes it very difficult to tease out the complex interactions between specific types of molecules and their effect on the final product.

A recent trend in beer brewing is the emergence of "hazy" pale ales, characterised by their intense hop aromatics, muted bitterness, and high turbidity, which causes their distinctive cloudy appearance. This turbidity, or "haze", is caused by complexes formed by excess proteins, starches, and tannins originating from the raw ingredients.[1] The increasing prevalence of this hazy beer style highlights the need to understand the yet-undetermined mechanisms responsible for the emulsification of aromatic terpenes from hops in hazy beers.[2] Given the greater stability of aromatic terpenes in hazy beer styles, a likely mechanism that merits further exploration is microencapsulation, where a water-soluble molecule (e.g. a protein) coats a hydrophobic molecule (e.g. hop oil), encapsulating it and allowing it to remain separate from the surrounding aqueous solution (e.g. beer). As a result, the encapsulated

molecules gain protection from destabilizing agents such as ultraviolet radiation, metals, and oxidation,[3] all known to contribute to early staling and flavour deterioration in beer.[4]

In cases where haze is not desired, brewers use stabilization techniques and preventative measures to create visual and sensory consistency. These techniques include cold conditioning to encourage particulate settling, filtration regimes, and the addition of enzymes or precipitants that target the proteins and/or polyphenols responsible for the majority of beer haze.[5] Two such commercial additives that specifically target gluten proteins and peptides are BrewTan and Brewers Clarex. BrewTan is a commercial tannic acid that forms an insoluble complex with haze-active proteins via hydrogen bonding. The complex then precipitates out of the solution. However, the solubility of the complexes can depend on the degree of polymerization, so smaller tannin-peptide complexes are formed but may remain soluble and pass into the finished beer.[6] Tannins also have the potential to alter the sensory profile of the finished product, especially if overdosed.[6,7] Brewers Clarex is a commercially available enzyme extracted from *Aspergillus niger* that contains a proline-specific endoprotease.[8] It is commonly used to combat a phenomenon known as "chill haze" (undesired protein-protein or protein-polyphenol aggregation[9]) by degrading proline-rich proteins such as gluten.[8,9] Brewers Clarex cleaves peptides and proteins, leaving behind smaller peptides that are less hydrophobic and less capable of forming the large protein-polyphenol complexes that contribute to beer haze[8] (Figure S1†). Furthermore, it has been shown to significantly reduce gluten mass and can target both large and small peptides with no impact on the sensory profile of the beer.[6] It is worth noting that we use "gluten" as the general name for this type of protein. In beer, barley does indeed contain a low percentage of gluten which most likely survives the brewing process. However, wheat contains glutenins and gliadins, and barley contains hordeins.

But how are hop oil emulsions in beer stabilised? While brewing grains naturally contain compounds capable of microencapsulation such as proteins and dextrins,[10–12] there is a lack of information in the brewing literature about their ability to emulsify and stabilise

hydrophobic substances, such as hop oils, in beer. Most current research in the brewing field on microencapsulation is limited to characterising haze-active proteins, starches, and tannins in beer.[13] Some studies have investigated the microencapsulation of brewing yeast for the primary alcohol fermentation of sparkling wine[14] and beer.[15,16] Earlier work investigated whether yeast encapsulation during fermentation affects the volatile chemical and sensory properties of the beer.[16] Later work used a similar method to microencapsulate yeast and carried out a more in-depth analysis of the volatile compounds produced and the sensory properties of the beer, finding that the product fermented by encapsulated yeast yielded a significantly different terpene and sensory profile than the unencapsulated yeast.[15] Notably, neither of these studies studies showed significant changes to the physical or chemical parameters (pH, alcohol content, bitterness, colour) of the finished beers fermented using encapsulated yeast, a desirable outcome when managing consistency in finished products.

Building on the extensive body of research on microencapsulation in the food sciences,[10] here we present a collaboration between academic researchers and industry brewing experts to explore this new frontier in beer brewing. We have developed a microfluidic platform to create oil-in-beer emulsions of controlled size and composition to investigate whether terpenes from hop oils in beer are stabilised by proteinaceous biomolecules. We test our hypothesis by exploring the effects of Brewers Clarex, a proline-specific endoprotease, on emulsion stability. Emulsions were created using beer as the aqueous phase and two hop terpenes (as the main components of hop oils) as the oil phase. Choosing two different hop terpene oils enabled us to determine whether structural differences between each oil had an impact on stability. The oil-in-beer emulsions created using our microfluidic platform were digested with different concentrations of Brewers Clarex, and the average number of droplets and average droplet volumes were monitored over time to assess the stability of the emulsions. Control experiments were performed to ensure that emulsion stability could be attributed solely to digestion with Brewers Clarex. These assays allowed us to investigate the role of proteins in the stabilisation of hop oils in beer and begin to answer this longstanding

question about beer.

Materials and methods

Materials

All reagents were used as received. Gluten from wheat, polyvinyl alcohol (PVA, 99%), chlorotrimethylsilane, sodium hydroxide, glacial acetic acid, linalool and α-pinene were purchased from Millipore Sigma. Acetate masks were printed at 10 μm resolution by CAD/Art Services. SU-83050 and developer were purchased from MicroChem. Silicon wafers (100 mm diameter) were purchased from Silicon Materials. Polydimethylsiloxane (PDMS, Dow Sylgard 184) base and curing agent (Sylgard 184 Silicone Elastomer Curing Agent) were purchased from Ellsworth Adhesives. Polytetrafluorethylene (PTFE) tubing (outer diameter 1/16 inch and inner diameter 0.75 mm) was purchased from Chromatographic Specialties. Brewers Clarex was a free sample from DSM Food Specialties. The beer (78 Kolsch, 5.0% ABV) was donated by Phillips Brewing and Malting Co.

Microfluidic device fabrication

Microfluidic platforms were designed using AutoCAD (Autodesk Student 2017 LTD) and printed onto acetate masks to create positive photomasks. The SU-8 layer was spin-coated onto the wafer and the wafer was soft baked for 5 min at 25 °C, 2 min at 65 °C and 30 min at 95 °C. Following the soft bake, the wafer was exposed to UV light for 11 s at 19.96 mW cm^2 using an OAI Model 800 mask aligner through the acetate photomask. The wafer was then heated at 95 °C for 15 min and cooled for 30 min. Unexposed SU-8 was removed using developer. Adhesion of the features was enhanced through exposure to UV light for 90 s at 19.96 mW cm^2 and a final hard bake at 200 °C for 30 min. The wafer was placed in a vacuum desiccator with 50 μL of vaporised chlorotrimethylsilane for 45 min to silanise the wafer and prevent sticking. PDMS base and curing agent in a 10:1 ratio were mixed

together thoroughly and poured over the silanised wafer, then placed in a desiccator under vacuum for 45 min to remove air bubbles. The PDMS-covered wafer was then placed in the oven at 65 °C to cure for a minimum of three hours to overnight. Once cured, the PDMS was carefully separated from the wafer mould and cut into individual devices using a scalpel. Access holes were punched using biopsy punches with a 1/16-inch outer diameter. PDMS was also spin coated onto glass microscope slides at 1200 rpm for 25 s and cured as above to create the bottom of the devices. Devices and PDMS-covered glass slides were washed thoroughly with soapy deionised water, isopropanol, and ethanol. The clean devices were placed in reverse osmosis water for 5 min, dried thoroughly with a filtered air gun, and then dried at 90 °C for 30 min on a hotplate. After the devices were dry, they were plasma bonded (Diener Electronic, Zepto ONE, 1 min, 100 W, 0.64 mbar) to the PDMS-covered glass slides.

Surface treatment of the microfluidic devices

The channels of the microfluidic devices were treated with a PVA solution as described previously[17] to ensure the production of oil-in-water emulsions. In brief, the PVA solution was made by dissolving 1.00 g of PVA in 100 mL of MilliQ water. The solution was stirred at room temperature for 45 min, then at 100 °C for 45 min, and then at 65 °C overnight. Immediately after plasma bonding the devices to the PDMS-covered slides, PVA solution was manually flowed through the channels using two gas-tight 1 mL glass syringes (Hamilton). The surface-treated devices were then placed in the oven at 65 °C for approximately 48 h before use.

Preparation of the beer aqueous phase

The beer used was a 375 mL canned Kölsch-style ale supplied by Phillips Brewing and Malting Co. Table S1† lists the specifications of this beer. Beer was obtained directly from the brewery and stored at 4 °C. A new can was used for each assay. The beer was from different batches due to the timeline of the project. This also minimised the effects of aging,

staling, and oxidation on our assays. Prior to insertion into the microfluidic device, the beer was degassed to ensure reproducible fluid dynamics on-chip. Beer was poured into a glass test tube and sonicated with a Fischer Analog Vortex sonicator. Once a foam layer formed it was removed from the liquid layer and the sonication step was repeated until there was no carbonation present in the beer.

Preparation of gluten solutions

As shown in Figure 1c, the aqueous phase on the microfluidic platform consisted of degassed beer and gluten at one of three different concentrations (no gluten, low gluten, high gluten). For the addition of gluten, first a 5% w/w gluten stock solution was made using gluten from wheat dissolved in 0.05% w/v NaOH solution and stirred at 55 °C for 30 min. It was allowed to cool, and 10% v/v glacial acetic acid was added. Table S2† shows the conditions tested to dissolve wheat gluten in 78 Kolsch beer without significantly changing the composition of the beer. We then acidified the dissolved gluten to ensure that the pH of the beer was not altered by the addition of the gluten solution (Table S3†). Figure S3† shows visually some of the conditions tested.

Serial dilutions were performed to reach a $2.5\text{x}10^{-5}$% (w/w) final gluten concentration and $2.5\text{x}10^{-1}$% (w/w) final gluten concentration for the low and high gluten assays, respectively. Table S4† shows how our gluten concentrations compare to those found in beer during the industrial production process. These solutions were added to the degassed beer prior to forming emulsions. When no gluten was added to the beer phase, the same amount of solvents used to make the gluten solution (but without the gluten) were added to the degassed beer prior to emulsion formation.

Creation of oil-in-beer emulsions

The design of the microfluidic device is seen in Figure 1a. A new microfluidic device was used for each experimental condition and for each repeat experiment. Hamilton Gastight glass

syringes (1 mL) were filled with hop oil and degassed beer to make oil-in-beer emulsions. Syringes were connected to the microfluidic device using PTFE tubing. The flow rate was controlled using neMESYS low pressure syringe pumps (Cetoni) with an initial flow rate of 15 μL/min for both phases, which was then adjusted to reach the highest throughput possible. Once equilibrated, the emulsions travelled thorough the device to the outlet where PTFE tubing carried the emulsions to a glass collection vial.

Brewers Clarex treatment of oil-in-beer emulsions

Oil-in-beer emulsions were collected in a glass vial until approximately 2 mL of emulsion was accumulated. Brewers Clarex was used as received for the high Brewers Clarex treatment and diluted to 50% v/v with reverse osmosis water for the low Brewers Clarex treatment. To prepare deactivated Brewers Clarex, it was heat-deactivated in a water bath at 70 °C for 15 min and then cooled to room temperature before use. Oil-in-beer emulsions were treated with these Brewers Clarex solutions as described in Table 3. Vials were then sealed and incubated in a water bath at 50 °C for 20 min prior to observation.

Droplet analysis

Droplet formation on the microfluidic devices was observed on a Nikon Eclipse Ti-U2 Inverted Research Microscope. Videos of emulsion formation and images of the on-chip emulsions were collected at 4x magnification using a Phantom high-speed camera (VEO 710L, Amatek). After 20 min of emulsion treatment as described in Table 3, emulsions were observed on microscope using a 40X/0.60 objective. Images were taken in the 4 quadrants of an observation slide made using Fisherbrand glass microscope slides and slide covers. For time series experiments, emulsions were sealed in a vial and stored in a 4 °C refrigerator. Images were taken again after 1 day and after 7 days. Images were processed using ImageJ (version 1.52a). Results showed a normal distribution and outliers were eliminated using the InterQuartile Range (IQR) Rule (if value $>$ or $<$ 1.5xIQR).

Results and discussion

Creating oil-in-beer emulsions

Microfluidic platforms allow control over individual droplet formation in an immiscible fluid stream, controlling droplet volume by regulating the relative flow rates of the two fluids. These technologies facilitate control over each emulsion component (oil phase, aqueous phase, emulsifier) which enabled us to design assays to tease out the interaction of specific classes of molecules within beer. Our microfluidic platform was designed to create oil-in-beer emulsions with a specific size and composition (Figure 1a). We used a flow-focusing junction with a pinched inlet and outlet to create the oil-in-beer droplets, and we collected them off-chip for digestion and analysis over the course of 7 days (Figure 1b). Figure 1c shows the composition of the oil (terpene) and aqueous (beer) phases. The oil phase consisted of either linalool or α-pinene, as two relevant terpenes present in finished beer.[18] The aqueous phase consisted of degassed beer to which was added all possible combinations of a protein (gluten) at different concentrations (none, low, and high) and/or an enzyme (Brewers Clarex) in different formats (none, active, and deactivated).

A useful approach to study the properties of an emulsion is to observe the stability of the oil-in-water mixtures over time while disrupting the suspected emulsifying agents. We chose to use a "clear" (transparent with no suspended particulate) Kölsch-style beer with low bitterness that had not been dry-hopped as the aqueous phase. This allowed for more control over the hop oil content in the emulsions we created using our microfluidic platform. Gluten was added to the beer prior to insertion into the microfluidic platform to mimic a hazy beer, and test whether this protein is one of the contributors to stabilising hop oils in beer. The oil-in-beer emulsions produced on-chip with different concentrations of gluten were then treated off-chip with the enzyme Brewers Clarex to determine whether digestion of the protein occurred. The oil-in-beer emulsions were analyzed for droplet volume and frequency post-digestion once they had spent 20 minutes in a water bath at 50 °C, since it is at this

temperature that the enzyme has 100% activity[19] (Figure S2 shows how we developed our incubation assays)†. Emulsions were then sealed and stored in the refrigerator at 4 °C until they were observed again after 1 day and after 7 days. Average oil droplet volume was used as a proxy for emulsion stability, since a stable average droplet volume over time indicates relative stability of the droplet population.

Gluten affects oil-in-beer emulsion stability

Our initial assays were designed to determine whether gluten affects the stability of oil-in-beer emulsions over time. Figure 2 shows how droplets in the assays change over time for oil-in-beer emulsions of α-pinene when subjected to three different gluten concentrations (no gluten, low and high concentrations of gluten). No Brewers Clarex was added to these assays. Figure 2a shows the average droplet volume, which was calculated by taking the mean of all droplet volumes recorded for each different treatment and monitoring over time. These values are shown in Figure 2b, which presents the data plotted as individual droplets, with green stars highlighting the mean, and Figure 2c shows the same assay data plotted as relative frequency, to highlight the number of droplets present in each assay at each time point.

Wheat gluten was chosen for these assays because it is readily available and is analogous to haze-active barley hordeins (which are a diverse class of proteins). In addition, wheat is frequently used as an adjunct in styles such as New England IPAs that desire haze formation. While not yet proven, the high prevalence of gluten in such styles is expected to help drive haze stability. Proteins in beer tend to be least soluble near their isoelectric points.[20] The isoelectric points of relevant gluten proteins from wheat are 4.6 (glutenin),[21] 5.5 (gliadin),[21] and the isoelectric point of gluten from barley (hordein) is 6.[20] The pH of the beer used in this study falls below the isoelectric points of the proteins (pH 4.22, Table S1†), indicating that a significant portion of proteins present in and added to the beer were solubilized.[22]

At the first measurement (Day 0), the emulsion with no gluten added (black data) had the

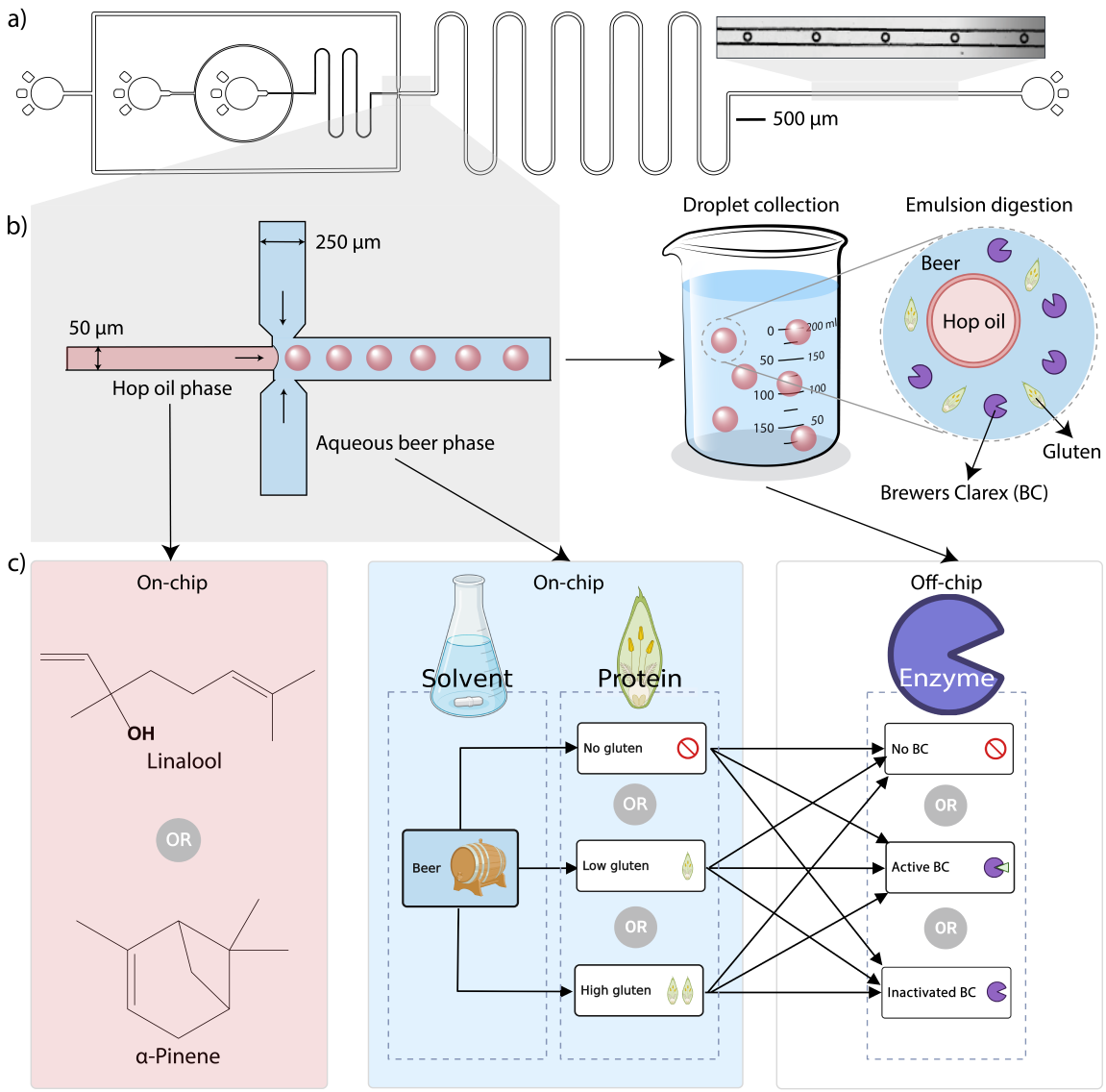

Figure 1: **Microfluidic platform and assay design.** a) Design of the microfluidic platform used for the formation of oil-in-beer emulsions. Emulsions were created in the flow focusing junction highlighted by a blue box. The other flow focusing junction was not used in these assays. A brightfield image of the oil-in-beer emulsions created is shown near the outlet of the microfluidic device. b) Dimensions and design of the flow focusing junction for the creation of hop oil droplets (red phase) in beer (blue phase), which sometimes also contains a protein (gluten). Emulsions were collected off-chip in a glass vial to allow for digestion with Brewers Clarex and analysis. The inset shows a graphical representation of the oil-in-beer emulsion after the addition of Brewers Clarex, where the red circle denotes the oil droplet, the green leaf represents gluten and the purple sector represents Brewers Clarex. c) Graphical representation of the assay, showing the composition of the hop oil and aqueous beer phases inserted into the microfluidic platform. The hop oil phase consisted of a terpene, either linalool or α-pinene. The aqueous phase is predominantly beer, to which a protein is added at two different concentrations (low or high gluten) on-chip. An enzyme is added in two different formats (active or deactivated Brewers Clarex) off-chip in all possible combinations. Control experiments where no protein or enzyme were added were also performed. Droplet frequency and volume were recorded directly after enzyme treatment (Day 0), after one day and after seven days. Image partially created with BioRender.com.

highest average droplet volume, with the addition of gluten at either concentration lowering this initial average volume (Figure 2a). After one day, this trend remained stable. After seven days, the low gluten (blue data) and high gluten (purple data) average droplet volumes had both changed, with the ANOVA between Day 0 and Day 7 showing a statistically significant difference in average droplet volumes ($p < 0.05$) for both gluten concentrations (Table 1). The emulsion not treated with gluten (black data) showed no statistically significant change in average droplet volume over time, suggesting that the emulsion remained stable over 7 days. Although both the low and high gluten treatments caused significant changes in average droplet volume over 7 days, the emulsion treated with a high concentration of gluten (purple data) changed more significantly and showed the highest change in average droplet volume, indicating that a high concentration of gluten may destabilise the emulsion more than a low concentration of gluten. When a low concentration of gluten was added to the emulsion, the average droplet volume stayed smallest in size overall over 7 days, but changed more significantly than when no gluten was added ($p = 0.65$). Overall, this indicates that the presence of gluten may contribute to emulsion stability over time and that this effect may also be sensitive to the amount of gluten present. This fits with anecdotal industry knowledge that beer styles with higher terpene content (such as pale ales) can be subject to more particulate coagulation, which can be a result of interactions between proteins and polyphenols.[23]

Table 1: One-way ANOVA data comparing the average droplet volume change between day 0 and day 7 for each gluten concentration (no gluten, low, or high gluten). These are the same data as shown in Figure 2a

	F value	**F statistic**	**p-value***
No gluten	0.43	3	0.65
Low gluten	124	3	<0.05
High gluten	16.8	3	<0.05

*$\alpha = 0.05$

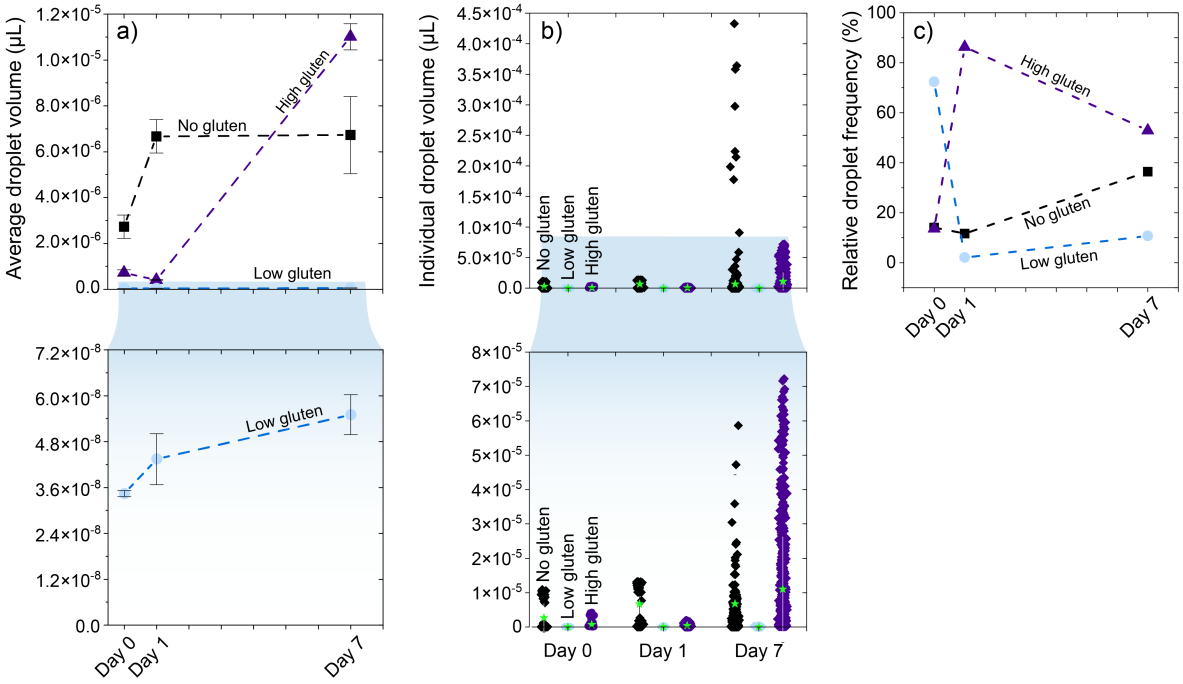

Figure 2: **Changes in droplet volume and frequency over time for α-pinene oil-in-beer emulsions treated with different gluten concentrations.** Oil-in-beer emulsions of α-pinene in beer treated with either no gluten (black data), low gluten (blue data), or high gluten (purple data) concentrations were monitored over seven days to assess droplet volume, a metric for how stable an emulsion is over time. a) Shows the average droplet volume at each time point (Day 0, Day 1 and Day 7). The inset below shows the same data for the low gluten assay using a smaller volume scale to enhance visualization of volume change over time. Lines are added between data points of the same assay to aid visualisation. b) Shows the spread in droplet volume for each condition shown in a), with the inset below showing the same data using a smaller volume scale to enhance visualization of the spread over time. Green stars show mean droplet volume for each time point. Error bars represent standard deviation (they are not always visible due to the large number of data points). c) Shows the relative droplet frequency for each time point. Lines are added between data points of the same assay to aid visualisation.

Brewers Clarex may stabilise hop emulsions

We then added Brewers Clarex, as an enzyme that digests gluten, to further test our hypothesis that gluten stabilises or destabilises hop oil in beer. Figure 3 shows oil-in-beer emulsions of α-pinene or linalool treated with the same gluten treatments as in Figure 2 (no gluten, and low and high concentrations of gluten), with the addition of four Brewers Clarex enzyme treatments: no Brewers Clarex, deactivated Brewers Clarex, low Brewers Clarex, and high Brewers Clarex. Figures 3a and 3c show the individual droplet volume of linalool or α-pinene emulsions when gluten and/or enzyme are added, and Figures 3b and 3d show the relative droplet frequency of the linalool or α-pinene emulsions under the same conditions. Deactivated Brewers Clarex was added as second control to determine whether other components of Brewers Clarex may affect emulsion stability. Table 2 lists the total

number of droplets in each data set in Figure 3.

When no gluten was added to the linalool emulsions (black data in Figure 3a), the mean individual droplet volume (green stars) stayed relatively stable upon treatment with deactivated, low or high concentrations of Brewers Clarex. However, the addition of deactivated Brewers Clarex caused a dramatic increase in droplet frequency (black data in Figure 3b), that is not present when either concentration of Brewers Clarex was added, which have a droplet frequency that is similar to when no Brewers Clarex is added. This may indicate that a non-enzymatic component of Brewers Clarex has a stabilizing effect on the emulsion stability.

In the α-pinene assays (Figures 3c and 3d) we see the effect that the molecular composition of the hop oil used has on emulsion stability. Linalool and α-pinene are only of one of several molecules found in hop essential oils in beer and have a low solubility in water, allowing us to make emulsions. Monoterpene alcohols such as linalool with a hydroxy group are more water-soluble than hydrocarbons such as α-pinene,[24] which could explain the difference in the spread in droplet sizes. When compared to the linalool emulsions, when deactivated or low concentrations of Brewers Clarex are added to α-pinene, we see increased stabilisation of the emulsion as shown by the low spread in droplet sizes (black data in Figure 3c). This suggests that there are multiple stabilisation mechanisms in effect, since the deactivated Brewers Clarex treatment did not affect the droplet frequency of the α-pinene emulsions in Figure 3d, or the spread in droplet volume in Figure 3c as it did in the linalool emulsions.

Individual droplet volumes for both linalool and α-pinene emulsions in Figures 3a and 3c did not change drastically with the addition of a low concentration of gluten (blue data). However, the relative frequency of droplets in both hop oil emulsions increased with the addition of both low and high Brewers Clarex doses (blue data in Figures 3b and 3d), indicating that the active enzyme influenced the frequency of droplets in the emulsions when a low concentration of gluten was present. When a high concentration of gluten (purple data)

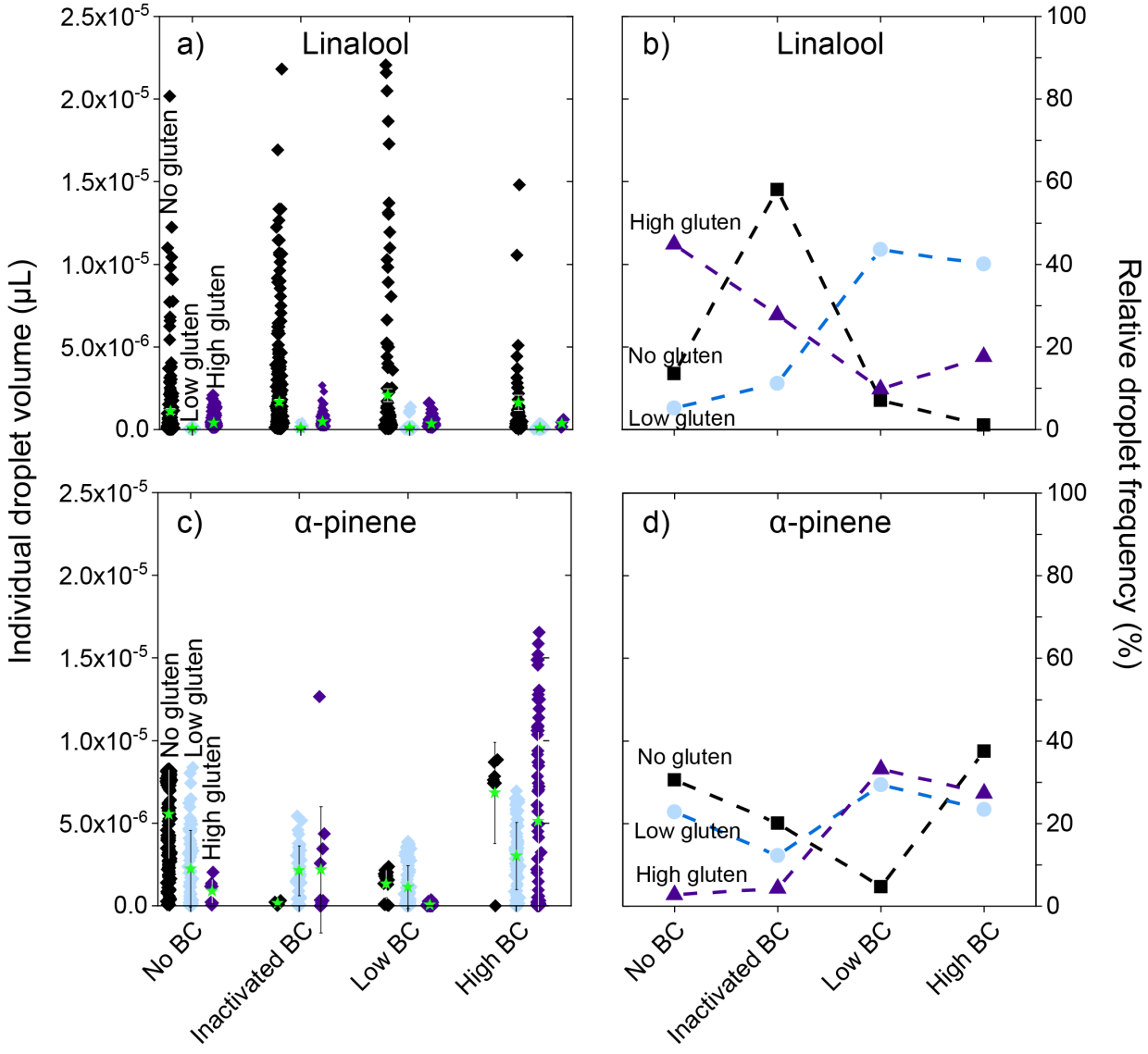

Figure 3: **Changes in individual droplet volume and relative droplet frequency of oil-in-beer emulsions upon the addition of Brewers Clarex.** Droplet volume changes when a) linalool and c) α-pinene emulsions are treated with different Brewers Clarex concentrations (no Brewers Clarex, deactivated Brewers Clarex, low concentration of Brewers Clarex and high concentration of Brewers Clarex). For each Brewers Clarex treatment, the emulsion also contains no additional gluten (black data), or low gluten (blue data) or high gluten (purple data) concentrations. This illustrates the changes in individual droplet volume upon the addition of gluten and/or enzyme for both types of oil-in-beer emulsions. Green stars show mean droplet volume for each treatment. Error bars represent standard deviation. Relative droplet frequency for each Brewers Clarex treatment illustrating the relationship between droplet frequency and the total volume of droplets for both b) linalool and d) α-pinene emulsions with no additional gluten (black data), or low gluten (blue data) or high gluten (purple data) concentrations.

Table 2: **Total number of droplets (n) included in each data set in Figure 3.** Each oil-in-beer emulsion of linalool or α-pinene had a protein added on-chip and an enzyme added off-chip in all possible combinations outlined in Figure 1

Linalool

Protein	**Enzyme**	**n total**
No Gluten	No BC	216
	Deactivated BC	427
	Low BC	158
	High BC	98
Low Gluten	No BC	24
	Deactivated BC	53
	Low BC	191
	High BC	207
High Gluten	No BC	286
	Deactivated BC	236
	Low BC	5
	High BC	129

α-pinene

Protein	**Enzyme**	**n total**
No Gluten	No BC	131
	Deactivated BC	86
	Low BC	161
	High BC	20
Low Gluten	No BC	84
	Deactivated BC	45
	Low BC	108
	High BC	86
High Gluten	No BC	7
	Deactivated BC	11
	Low BC	85
	High BC	70

was added to the oil-in-beer emulsions, we saw contrary influences between the two terpenes, with a decrease in droplet frequency for linalool emulsions treated with low and high Brewers Clarex doses (purple data in Figure 3b), and an increase in droplet frequency for α-pinene emulsions treated with low and high Brewers Clarex doses (purple data in Figure 3d). The mean droplet volume (green stars) of linalool emulsions with a high gluten concentration did not change when treated with deactivated, high, or low Brewers Clarex doses (purple Figure 3a), while α-pinene emulsions showed dramatic increases in both mean volume and range of droplet volume sizes when a high dose of Brewers Clarex was used (purple Figure 3c). This suggests that gluten may influence the emulsion stability of certain hop oil emulsions such

as α-pinene, but not others such as linalool. It remains to be determined whether this trend would hold across hydrocarbon and monoterpene alcohol groups.

Overall, oil-in-beer emulsions of α-pinene or linalool with a low concentration of gluten present (blue data) treated with a low concentration of Brewers Clarex showed the smallest individual droplet volume and highest relative droplet frequency, indicating that in "hazy" style beers where there are more haze-active proteins present than in "clear" style beers, a low concentration of Brewers Clarex may serve to stabilise hop oil emulsions. The deactivated Brewers Clarex treatment groups had individual droplet volumes and relative droplet frequencies different than the no Brewers Clarex treatment groups, suggesting other components of Brewers Clarex had an impact on emulsion stability. Finally, different structures of the hop oils in the emulsion may influence the emulsion stability, which could have ramifications for brewers choosing which hops to include in "hazy" style beer recipes when aiming for a shelf-stable brew.

Table 3: **Oil-in-beer emulsion treatments of samples used in Figure 3.** In each case 100 µL of beer and either α-pinene or linalool oil-in-beer emulsions were collected and treated off-chip with the solutions described in the column labelled "Off-chip treatment". In the case of the "No Brewers Clarex" sample, an extra 100 µL of beer and either α-pinene or linalool oil-in-beer emulsions were added to ensure volume consistency. Likewise, in the case of the "Low Brewers Clarex" sample, an extra 50 µL of beer and either α-pinene or linalool oil-in-beer emulsions were added to ensure volume consistency

	Off-chip treatment	**Final treatment concentration**
No Brewers Clarex	100 µL of oil-in-beer emulsions	Pure oil-in-beer emulsions
Deactivated Brewers Clarex	100 µL of deactivated Brewers Clarex	50% (v/v) deactivated Brewers Clarex
High Brewers Clarex	100 µL of Brewers Clarex	50% (v/v) Brewers Clarex
Low Brewers Clarex	50 µL of Brewers Clarex and 50 µL of oil-in-beer emulsions	25% (v/v) Brewers Clarex

The use of microfluidic technologies to study emulsion properties is an innovative and underused tool for revealing key emulsion constituents in the beverage industries. We have shown that using a microfluidic platform to digest oil-in-beer emulsions in the presence and absence of gluten and Brewers Clarex is a rapid and effective way to assess the stability of the emulsions under multiple conditions that reflect the full-scale conditions found in the brewing industry. Oil-in-beer emulsions were affected by the addition of gluten to the emulsion, along with Brewers Clarex, a proline-specific endoprotease known to efficiently digest gluten in beer. Both α-pinene and linalool emulsions with a low concentration of gluten present showed higher stability in droplet volume and frequency when treated with

a low concentration of Brewers Clarex, though it is not yet clear if the active enzyme or another component of Brewers Clarex was responsible for the overall emulsion stability.

The application of microfluidic platforms in the food sciences is still in its infancy, and as such, this early work inspires many further questions. Future work in understanding how proteins affect hop oil emulsion stability could be done using the new methods we have developed, with other common molecules found in beer originating in brewing grains. Barley gluten was not commercially available for this work, but future work could seek to include haze-active barley hordeins that mirror more closely the composition of this beer style. We suggest also using starch, as a polysaccharide commonly found in conjunction with gluten and enzymatically processed by amyloglucosidase during brewing. It would also be interesting to test whether glycogens and dextrins have a stabilising effect on hop oil droplets in beer. Starch and dextrins broadly form many different types of complexes in solution, including cyclodextrins which are known to encapsulate and stabilize oils.

We also suggest that using a wider panel of hop oils, including a selection of both hydrocarbon- and monoterpene-structured hop oils, could further elucidate whether the structure of the individual hop molecules affect how stable they are when emulsified in beer. This could also lead to future work involving analysis of the individual molecular components of the oil-in-beer emulsion as it ages, to assess if structural changes in individual molecules contribute to overall emulsion stability. The impacts of the grain contributing gluten, including wheat,[25] as well as storage temperature of the beer[26] are also of interest for commercial operations seeking to optimize visual and aromatic shelf stability. A final area of interest would be to include experimental conditions that mimic dry hopping,[27–29] and the many chemical interactions created that may affect hop oil stability in beer.

This work and future studies based on it may lead to interesting conclusions for industrial brewing. If certain hop oils in conjunction with adjusted proteinaceous components of beer are more stable over time while not sacrificing sensory qualities of the final product, brewers could adjust recipes to create beers that better withstand the aging process. Work has al-

ready been done on individual hop oils and their respective shelf stabilities.[30,31] Meanwhile, specific haze-active proteins have been identified from barley.[32] Combining these findings with ours offers an opportunity to model the specific interactions in haze and emulsification chemistry in a microfluidic platform to reveal what this means for the hazy beer brewing process for recipes yet to be developed, and drank by brewers, scientists, and market consumers alike.

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Authors contributions

ARM and JLK gathered the data presented here, except where otherwise noted below. KR developed the microfluidic device, the surface treatment protocol, created Figure 1, and edited the introduction. NY developed the protein solubilization method and made Table S2, Table S3, and Figure S3. DH developed the droplet incubation timing and collected the data for Figure S2. JK and CSS performed preliminary experiments. AS processed all ImageJ data. ARM analyzed all data and wrote the first draft of the manuscript, except

for the first draft of the introduction (written by ELT) and the first draft of parts of the Materials and Methods (written by DH). ELT and KSE conceived the experiments. KSE edited the manuscript and supervised ARM, DH, JK, JLK, KR, AS, CSS and NY. DH, JK, KR, AS, CSS and NY appear in alphabetical order as authors.

Supporting Information Available

Electronic Supplementary Information (ESI) available: incubation conditions for the enzyme assay, specifications of the 78 Kolsch beer, conditions tested to solubilise and acidify gluten, and comparison of gluten concentrations used here with industry values.

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