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Using lipase activity to probe milkfat globule membranes (MFGM) or interfaces

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ABSTRACT

Measurement of lipoprotein lipase activity (as free fatty acid change or accumulation) is accepted as indicative of milkfat globule membrane (MFGM) disruption. However, measurement is confounded by variables unrelated to the MFGM, e.g., microbial quality. To resolve this, a modified approach to using lipase activity to probe MFGM was developed. Methanol (1%, w/w) and lipoprotein lipase (2%, w/w, raw milk) were added to pasteurised milk samples resulting in formation of methyl esters that therefore served as a new and unique signal for lipase gaining access to interfacial fat. Analysis conditions minimised product formation to minimise sample perturbation. The method could detect 1% (w/w) of homogenised milkfat globules blended with native globules, with linear response to 10% (w/w) and saturation above 20% (w/w). The approach was applied to assess MFGM in samples subjected to disruptive treatments (shear, aeration) under different conditions; findings were consistent with previous research, but with greater sensitivity.

1. Introduction

Dairy processing starts with raw whole milk containing milkfat globules (MFGs). The behaviour of MFG is an important consideration for achieving desired products, even if the only requirement is MFG removal (e.g., centrifugation to produce skim milk). Therefore, methods to characterise structures and properties of MFG have always had strong demand.

Native MFG, when secreted from mammary glands, have a highly defined structure (Evers, 2004a; Singh & Gallier, 2017), including a surface-active interfacial tri-layer called the milkfat globule membrane (MFGM). From secretion onwards, MFG and the MFGM are susceptible to changes in structures and properties, whether intended or not (Evers, 2004a). When the MFGM is sufficiently different from its native state, a more generic and appropriate term such as 'interface' is used to describe the surface-active layer, often mono-layer, of a milkfat droplet.

The changes just described could be referred to as 'damage' if the context is that changes are undesired or unintended (or both; Evers, 2004a). However, from a different perspective, what was considered 'damage' might be deemed 'optimal processing'.

Often interfacial (i.e., MFGM) changes are linked to other changes in emulsion structure (i.e., MFG), such as size distribution. For accuracy, in this paper the term MFG is used when interfacial and non-interfacial phenomena (e.g., size changes) are indistinguishable; the use of the term MFGM is reserved specifically for discussion of interfacial causes or effects.

The purpose of this paper is not to classify changes as 'damage' or 'optimal processing' for MFG or MFGM, but to outline a partial means of probing changes in interfacial or related properties, regardless of their consequences. This is because there is a requirement for simple, sensitive, and minimally perturbing methods to characterise interfacial property changes in milkfat (o/w) emulsions (Evers, 2004b). Such methods could either measure interfacial properties directly or use a probe, where the signal of the probe changes in response to changes in the interface.

Lipases are enzymes that operate at water/lipid interfaces to catalyse lipolysis and therefore have repeatedly demonstrated potential suitability as interfacial probes (Deeth, 2006; Evers, 2004b). That is, lipases are well known for being active (i.e., signalling) when specific interfacial properties occur, but are otherwise inactive (i.e., not signalling). Naturally, 'signals' produced by enzymes, including lipases, are amplified because one active enzyme molecule will catalyse the conversion of many substrate molecules to product molecules.

A well-known example of lipase responding to changes in interfacial properties is lipoprotein lipase (LPL). LPL is abundant in

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raw milk, but is essentially inactive (i.e., not signalling) if interfacial properties are minimally different from that of native MFGM at secretion (Deeth, 2006). When aeration or shear (or both) changes interfacial properties (i.e., 'disrupts MFGM'), LPL becomes progressively more active (i.e., greater signalling) producing accumulating amounts of free fatty acids (FFAs). Thus, for many years, LPL has been used as a crude probe for cumulative changes in milkfat interfacial properties signalled through FFA increases. In a similar manner, pancreatic (Mun, Decker, & McClements, 2007) and fungal lipases (Deeth & Fitz-gerald, 1978) were shown to exhibit lipase activity differences on different interfaces.

The interfacial properties to which lipases respond are not fully characterised and will depend on the origin of the lipase (Verger, 1997). However, for insoluble substrates, a lipase can only be active when correctly positioned at an oil/water interface, which means it must displace other interfacial components. Thus, lipase activity indicates something about the resistance of an interface to displacement by lipase, as determined by a combination of, e.g., charge, stearic factors, interfacial tension, etc. (Danthine & Blecker, 2014).

To summarise, in theory it appears straight-forward to use lipases to probe interfaces because they will signal (amplified) when at an interface and otherwise will not, but to make this work in practice can be difficult. This is primarily because the signal created by a lipase at an interface (lipase activity) is difficult to measure without confounding factors (Evers, 2004b); lipase activity depends on temperature, pH and enzyme properties, which could all be confounded with interfacial effects.

Nevertheless, Iametti, Versuraro, Tragna, Giangiacomo, and Bonomi (1997) provided a comprehensive example of how lipase can be used to probe interfaces by relating lipase activity to other interfacial characteristics. They found that using dyes to probe interfacial hydrophobicity was ineffective while probing with lipases was effective; the superiority of the latter was probably due to the inherent amplification associated with an enzyme-based measurement. However, their work did not extend to determining the limits in sensitivity of using lipases as probes, which would have been low due to the use of a pH-stat method.

In this paper, the ideas of Iametti et al. (1997) and prior authors (Deeth & Fitz-gerald, 1978), are taken a step further to determine how sensitive a lipase could be as an interfacial probe. A method is proposed to use lipases to probe interfacial properties using a recently developed indirect measurement of lipase activity (Andrewes, 2018) that makes it possible to minimise confounding factors. Critically, a new nucleophilic reagent (methanol) is added to samples so that active lipases at interfaces generate a new signal (methyl ester formation) distinguishable from any previously formed FFAs. As discussed previously (Andrewes, 2018), because methanol is an alternative nucleophile to water, methanol only plays a role after the enzyme has bound to the substrate, consistent with established mechanisms for lipase action (Beer, Wohlfahrt, McCarthy, Schomburg, & Schmid, 1996). Thus, interfacial lipid continues to be the substrate for the enzyme and indirectly measured activity reflects this.

Measuring activity as methyl ester formation serves to eliminate any background FFAs, but also gives the assay sufficient sensitivity so that enzymes and internal standards can be added in a manner that minimally perturbs the sample being evaluated. Sensitivity coupled with automation also allows high throughput enabling numerous negative and positive controls to be analysed to correct for confounding factors. Additionally, the approach is designed with an emphasis on simplicity to minimise possibilities for error.

The proposed approach is demonstrated by examining the effects of homogenisation and aeration on MFG from diluted pasteurised cream, including comparisons with prior literature (Deeth, 2006; Deeth & Fitz-gerald, 1978; Iametti et al., 1997). Diluted cream (~4% fat) was chosen for the initial research because assay sensitivity was best at low fat content and the MFG were in a simple matrix; however, matrix complexity was progressively increased to demonstrate potential for future experiments to apply this approach to industry-relevant research (e.g., impact of milk pumping on final product properties due to MFG disruption).

2. Materials and methods

2.1. Materials

Raw milk was collected from a farm in Manawatu, New Zealand, milking a mixed herd of approximately 300 cows following organic grass-fed practices. Collection was in mid-Winter. Milk was divided into aliquots and frozen (−20 °C) to use as a consistent source of lipoprotein lipase (LPL) for all experiments. Due to the freezing, the milk is also expected to be a source of disrupted MFG and, therefore, background lipase activity. However, on-going monitoring of negative and positive controls gave consistent, acceptable results indicating defrosted raw milk (at 2%, w/w) was a consistent source of LPL with minimal background activity until additional disrupted MFG are added.

Lipase from *Pseudomonas cepacia* was in the form of a powder from Sigma Aldrich (St. Louis, MO, USA) freshly reconstituted to 100 mg L^{-1}. This solution was added to samples at 2% (w/w) to obtain results described in Section 3.6.

Cream and whole milk (not homogenised) samples were pasteurised (i.e. LPL was deactivated) commercially-manufactured products (38% fat cream, and 4% fat milk) purchased locally (Anchor Milk, Palmerston North, New Zealand). Small fat plugs (obviously containing damaged fat globules) found in containers of cream were discarded before taking samples.

2.2. Assay

All samples were analysed in the same manner. Three types of analyses were used in all experiments: (i) negative controls, where lipase activity of a sample was measured without adding lipase; (ii) samples measured after addition of a lipase, usually in the form of 2% (w/w) raw milk, unless otherwise stated; (iii) positive controls, where a sample contained a known amount of homogenised material, as an internal or external standard, defined as having a relative lipase activity of 1.

Samples above were prepared and methanol (1%, w/w, ACS reagent grade, Merck, St. Louis, MO, USA) added to initiate formation of methyl esters due to any lipase activity present. Samples were immediately placed in a temperature-controlled autosampler rack (held at 10 °C, except as described in Section 3.5) to be analysed as soon as their position in the analysis queue was reached. Triplicate analyses led to incubation times of approximately 1–8 h, 5–12 h, or 10–18 h. Exact incubation times were noted as data-file time-stamps.

All further details of analysis were as previously described (Andrewes, 2018), except for the conditions for headspace solid-phase microextraction (SPME). For this work, SPME extraction was directly performed in the autosampler rack so that sample temperatures were constant during both incubation (i.e., awaiting analysis) and analysis (i.e., SPME extraction).

Where necessary (Section 3.5), to prevent microbial growth, sodium azide (0.02%, w/w, Sigma Aldrich) was added to samples.

2.3. Preparation of samples for assay

For all but the final set of experiments, diluted cream (10%, w/w, cream in chilled water) was used as a source of MFG that was assumed to be minimally disrupted. To disrupt MFG, diluted cream

was either warmed to approximately 50 °C or kept at its current temperature, after dilution (<10 °C), and subject to shear or aeration (and shear) as follows.

Cream was homogenised (shear) using a laboratory-scale homogeniser (GEA Niro Soavi, Panda Plus; GEA, Parma, Italy) only using the first-stage homogeniser valve to control pressure. Negative controls were material pumped through the homogeniser with pressure set to zero. Otherwise, samples were homogenised at the pressures specified in the results, and, as needed, the pressure increased step-wise to collect samples at each of the stated pressures. Unless stated otherwise, the homogenisation pressure was 30 MPa (the highest pressure used in this study).

Samples for whipping (aeration and shear) were prepared in the same way as samples for homogenising. Samples (400 mL) were whipped using a Hobart planetary mixer and a D-wire whip (Model N50, Hobart Corp., Troy, OH, USA). Samples were 'lightly whipped' by using speed setting 1 (136 rpm at agitator, 60 rpm at attachment) for 30 s. This produced very few visible bubbles. Samples were 'moderately whipped' by using speed setting 3 (580 rpm at agitator, 255 rpm at attachment) for 60 s, producing many visible bubbles (i.e., foam). To produce an 'extensively whipped' sample, the highest-speed mixing was continued for a further 2 min.

For experiments reported in Section 3.7 samples (400 mL), at less than 10 °C, were whipped at speed setting 3 for 1 min. This experiment used an internal standard where 10% (w/w) internal standard was added to a sample. The internal standard contained diluted cream (50 °C) homogenised at 30 MPa. Therefore, samples containing internal standard would contain ~0.4% (w/w) disrupted MFG and ~3.6% MFG from the sample.

2.4. Statistical analysis and experimental design

Simplified principles of method development and validation were followed, as laid out in the Eurachem guide (Magnusson & Örnemark, 2014). The extent of validation chosen was consistent with research requirements rather than regulatory requirements. All samples and analyses were performed in triplicate unless otherwise stated. Where appropriate, the ability of the method to detect differences in sample treatments was tested using analysis of variance (ANOVA) using Minitab software (version 17.2, Minitab Inc., State College, PA, USA) and a significance level of $p < 0.05$.

3. Results and discussion

For all experiments, multiple methyl esters (e.g., methyl butanoate, methyl hexanoate and methyl octanoate) were measured in samples. However, instrument responses for different esters were highly correlated, as expected. Therefore, results reported are only those for methyl hexanoate, as representative of all esters.

3.1. Determining optimal conditions for probing interfaces using a lipase

In a preliminary experiment (not shown), lipase activities in creams (~40% fat) and whole milks (~4% fat) were compared for both native MFG (unhomogenised) and intentionally disrupted MFG (homogenised). While discrimination of MFG was possible at both 40% and 4% fat, instrument sensitivity was approximately 10-fold higher for 4% fat samples. This is because, using SPME, esters in creams are difficult to recover due to partitioning into fat. Thus, all further experiments were done on samples containing 4% fat. However, diluted cream, not whole milk, was used as the source of native MFG to achieve a simpler matrix.

To demonstrate the approach to using a lipase to probe an interface, raw milk was used as a source of lipoprotein lipase (all Sections, except 3.6). The same approach, demonstrated here, can be used with other choices of lipases as probes; the choice of LPL for these pieces of work was influenced by the relevance of LPL to the dairy industry and LPL's known ability to differentiate native MFGM from other interfaces (Deeth, 2006).

The objective of this section of work was to find optimum conditions that adequately differentiated samples, based on interfacial differences, while minimising perturbation of samples. The formation of surface-active products (esters, FFAs and mono- and diglycerides) due to the action of an enzyme at an interface can be considered a perturbation of a sample. Therefore, identifying experimental conditions causing minimal product formation was an objective of this optimisation.

Low temperatures minimise product formation, maximise in vitro enzyme activity relative to non-enzymatic reactions (Van Boekel, 2008), and keep confounding microbial growth in control. Therefore, samples were incubated at only 10 °C (the lowest feasible temperature for the equipment available) for this work, but temperature is expected to change conditions at interfaces, and so is explored in more detail later (Section 3.5).

Product formation was also expected to be proportional to enzyme concentration and incubation time, where both variables are related in a way (Michaelis–Menten kinetics) whereby one or the other can be changed to give the same effect. However, it was more practical to control enzyme concentration while incubation time defaulted to a known (but not controlled) value determined by a sample's position in the analysis queue; samples that were being compared were placed next to each other in the queue so that their incubation times were essentially the same. Furthermore, by limiting incubation to the time in the automated analysis queue, rapid results are produced, providing an advantage over other methods.

The data for optimising raw milk LPL as a probe are shown in Table 1. The highest concentration of LPL assessed (20% raw milk in the sample) readily distinguished [with good repeatability: 2% relative standard deviation (RSD)] a homogenised sample (new interface) from its control (native MFG). The differentiation of samples was independent of the time samples were incubated. This was because both the homogenised sample and its control form esters in proportion to incubation time, but incubation time effectively cancels out when samples are compared. In all subsequent work, incubation time is eliminated as a variable by always comparing samples with appropriate positive controls (standards) where both samples receive approximately the same incubation time.

Errors, due to assuming equivalent incubation times are expected to average near 20% (1 h difference between compared samples after 5 h incubation). Such errors are at least as good as reported for alternative approaches (Iametti et al., 1997). Furthermore, the impact of the error decreases as samples are incubated for longer, which was apparent from analysis of repeats. Thus, error bars in later presented results (particularly Fig. 2) represent the worst-case, where the first repeat deviated from consistent 2nd and 3rd repeats due to timing errors. This approach does not affect the conclusions from this work but suggests options for future improvement, if needed.

Decreasing the concentration of the probe (2% raw milk in the sample) only decreased the differentiation between samples if the incubation time was very short. With decreased amount of enzyme and very short incubation there is little product formed, so noise in the analysis and timing errors become significant. The required incubation to get good results is such that most samples will achieve the requirement, by default, as they sit in the analysis queue.

A further decrease in the concentration of the probe (0.2% raw milk in the sample) gives comparable differentiation in samples,

Table 1
Lipase activity, as methyl hexanoate formation, measured in intact milk fat globules (i.e., diluted cream) compared with globules disrupted by homogenisation at 30 MPa.[a]

Incubation time	Lipase probe (%)	Absolute lipase activity		Differentiation	RSD (differentiation)
		Intact MFG	Homogenised MFG		
Short	0	6095	19,776	3.2	
Medium	0	5885	29,029	4.9	
Long	0	4871	38,109	7.8	43%
Short	0.2	9492	37,529	4.0	
Medium	0.2	10,531	91,692	8.7	
Long	0.2	12,627	148,934	11.8	48%
Short	2	37,366	269,048	7.2	
Medium	2	55,477	701,519	12.6	
Long	2	78,308	1,039,687	13.3	30%
Short	20	167,509	1,973,845	11.8	
Medium	20	292,765	3,560,273	12.2	
Long	20	383,132	4,705,756	12.3	2%

[a] Measurements were made under the different conditions tabulated to identify requirements for maximum differentiation of the two sample types, with consistency in differentiation (minimal RSD) while minimising product formation. Lipase probe is the amount of raw milk used, as a source of lipoprotein lipase (%, w/w). The peak area for methyl hexanoate was that measured after incubation (from wait in analysis queue). Differentiation is the lipase activity for homogenised MFG divided by lipase activity for intact MFG; RSD, relative standard deviation.

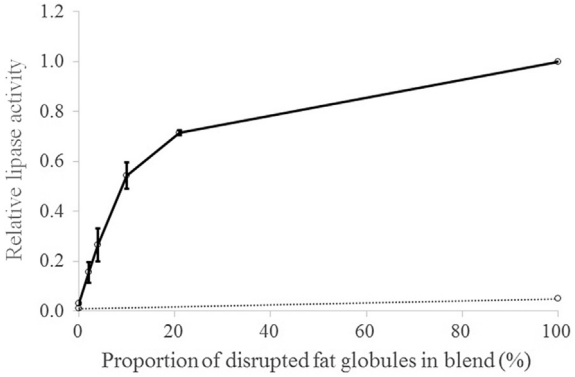

Fig. 1. Relative lipase activity in blends of disrupted (homogenised at 50 °C, diluted cream) and native (diluted cream) milk fat globules. All samples contained 4% fat. Error bars represent ±1 standard deviation for 3 repeats, and where not visible were less than the symbol size. A sample homogenised at 30 MPa, and 50 °C, was defined as having a relative activity of 1. Activity was measured without adding lipase (---) or in presence of lipoprotein lipase (——).

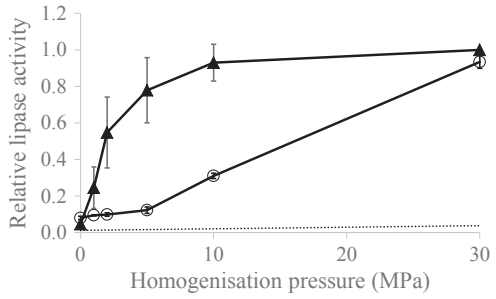

Fig. 2. Relative lipase activity measured in diluted cream (4% fat) after milk fat globules were disrupted by sample homogenisation at different pressures and at a temperature of 10 °C (◯) or 50 °C (▲). Error bars represent ±1 standard deviation for 3 repeats, and where not visible were less than the symbol size. A sample homogenised at 30 MPa, and 50 °C, was defined as having a relative activity of 1. Activity was measured without adding lipase (---) or in presence of lipoprotein lipase (——).

but only with very long (12 h) incubation and poor reproducibility. Thus, the use of 2% raw milk as a source of lipase was selected as the best compromise between achieving differentiating lipase activity and minimising perturbation of the sample. Under these conditions changes in fat, proteins and pH, attributed to the raw milk (i.e., 2% of the sample), should be minimal and readily corrected for using controls. The appropriateness of this choice is further verified in subsequent sections of this paper.

It was interesting to find some distinction of samples without the addition of lipase; this might be due to traces of lipase in the samples before LPL was added. An alternative explanation is that the non-enzymatic reaction rate for methanol and fat is also likely to be dependent on interfacial conditions; however, enzymes, as catalysts, amplify what happens at interfaces and therefore are expected to produce more readily measured signals compared with chemical probes (i.e., interfacial dyes or methanol alone) that lack a mechanism for producing an amplified signal.

3.2. Effect of substrate ('disrupted' MFG) concentration on relative lipase activity

The amount of 'disrupted' MFG in samples was systematically changed by blending a homogenised sample ('disrupted' MFG) with its control (native MFG). This approach ensured that all samples contained the same composition originating from the same source material (diluted cream). The relationship between the enzyme activity and interfacial changes, expressed as percentage homogenised MFG in the blend is shown in Fig. 1. Enzyme activity results are expressed relative to a positive control (that contained 100% homogenised material) to correct for incubation time (and other non-interfacial variables).

The detection limit (3 standard deviations from the control) for this experiment was determined to be 1% homogenised MFG in a blend. Thus, a linear discrimination, based on interfacial composition, was possible for 1–10% blends, but discrimination of samples was poor for blends between 20% and 100%.

Such saturating behaviour was consistent with Michaelis–Menten kinetics (Van Boekel, 2008), where at high substrate concentration enzyme turn-over becomes limiting. That is, the amount of substrate for the enzyme is not the amount of fat (constant 4% for all samples) but the amount of interfacial fat accessible to the enzyme. Thus, the intention of this assay was to measure the amount of substrate (by calibration against standards) where substrate represents 'disrupted' interfacial material. However, Michaelis–Menten kinetics implies that the ability to

distinguish differences in substrate concentrations decreases as the substrate concentration increases to a point of saturation.

Such saturation is not necessarily a problem as most researchers are not interested in methods to characterise highly processed samples (Evers, 2004b); however, in theory, for blends above 20% better discrimination of samples could be obtained by diluting samples.

3.3. Effect of hot and cold homogenisation on interfaces as measured by relative lipase activity

As a first demonstration of the application of this assay, either hot (50 °C) or cold (10 °C) diluted cream was passed through a homogeniser at different pressures. Relative lipase activity measured in these samples as a function of homogenisation pressure is shown in Fig. 2.

Similar profiles for Fig. 1 (blending) and 2 (hot homogenising) indicate that the saturation behaviour occurring in Fig. 2 as hot homogenisation pressure increases is due to substrate saturation (Michaelis–Menten) kinetics, rather than homogenisation causing less change at higher pressures. That is, calibration of Fig. 2, based on Fig. 1 indicates that the quantity of 'disrupted' interface is directly proportional to the homogenisation pressure (when hot).

The large error bars in Fig. 2 (hot homogenising) appear to be an artefact due to assuming a sample and its standard receive the same incubation when they do not, the error being attributed to the first set of repeats with the shortest incubation time. Errors in Fig. 1 were also mostly caused by the first repeat, not the second and third repeats. However, it is not clear why the errors were larger in Fig. 2 for hot homogenising compared with cold homogenising and with blending (Fig. 1). It is suspected that the enzyme kinetics might deviate from Michaelis–Menten in certain situations, possibly when lipase activity at an interface disrupts the interface. Alternatively, subtle changes in analysis of esters in different types of samples may not be fully corrected for using the positive control. However, because there is no clear reason to reject data from short incubations, even though they appear to cause slightly anomalous results, they have been included to represent a worst-case scenario and conclusions are kept appropriately conservative.

At cold temperatures homogenisation at low pressures causes a measurable change in samples that is almost independent of homogenisation pressure up to 5 MPa (Fig. 2). Above 5 MPa, the effect of homogenisation rapidly increases until at very high pressures there is little distinction between hot and cold homogenisation (partly because the assay on the hot sample is at saturation).

Findings in this section are generally consistent with what is known about effects of homogenisation on MFG, interfaces, and, therefore, lipase activity (Deeth, 2006; Iametti et al., 1997), so supporting the validity of this assay. However, previous studies often only evaluated extreme conditions because those assays were not sufficiently sensitive to obtain finer detail.

To explain detailed findings, at cold temperatures some speculation is required. It is hypothesised that, for pressures up to 5 MPa, the only material likely to be disrupted will be bigger than the narrowest gap in the homogeniser valve that must break or else the valve would block. The valve gap minimally changes with increasing pressure, so no changes occur in the amount of material disrupted. However, once a pressure of 10 MPa is reached, turbulent forces become sufficient to disrupt smaller particles. At hot temperatures, where all material is liquid, turbulence should play a much greater role at all pressures.

Here and in the previous section the activity measured is an average for a population of lipases acting across numerous MFG. But, clearly in each section population distributions will be different. Homogenised samples (this Section) are likely to have many slightly disrupted MFG while blended samples (previous Section) are more likely to only contain a few severely disrupted MFG. Thus, caution is required because this method cannot infer population distributions of MFG variants. This is a problem shared by any measurement of MFG based on bulk properties. Understanding population distributions (after measuring averages) is only possible through corroborating evidence. As this paper is focused on method development, such corroborating evidence is not considered.

3.4. Effect of hot and cold whipping on interfaces as measured by relative lipase activity

It is well known that the combination of aeration and shear, such as created by whipping equipment, profoundly effects MFGM (Deeth & Fitz-gerald, 1977, 1978). Thus, to further demonstrate application of this assay, diluted cream was assessed after different whipping regimes (Fig. 3). As expected, the whipping process caused changes in lipase activity that could be measured.

In contrast to homogenisation, the effect of temperature on the amount of change was not significant. It was also observed that the gentle whipping scenario produced few visible bubbles (or foam) and no detectable increase in lipase activity, whereas a faster whipping speed, creating visible bubbles (and foam), did result in an increase in lipase activity. These findings are consistent with earlier work (Deeth, 2006). In this experiment the likely cause of MFG change was flow of MFGM material onto water-air surfaces of bubbles (Evers, 2004a). This process would be expected to be somewhat temperature independent, whereas confounding shear forces should have more impact at hot temperatures (based on Section 3.3). At cold temperatures partial coalescence can also occur leading to increased particle size and ready detection by size measurement. However, at hot temperatures, partial coalescence and associated particle size changes is unlikely, even though air bubbles will change MFGM. Therefore, this type of assay could be most applicable to detecting changes in interfaces, for constant particle size, caused by aeration when milk is above 40 °C (e.g., during milking or pasteurisation).

3.5. Effect of temperature on interfaces as measured by relative lipase activity

When developing lipase assays based on ester analysis, there was no advantage in operating at high temperatures to form more

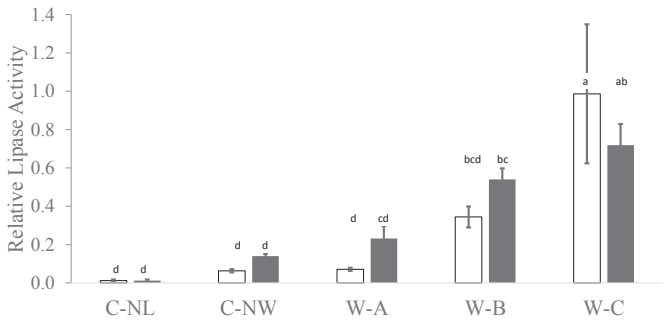

Fig. 3. Relative lipase activity measured in diluted cream (4% fat) after milk fat globules were disrupted by whipping at sample temperatures of 10 °C (□) or 50 °C (■). Error bars represent ±1 standard deviation for 3 repeats. A sample homogenised at 30 MPa, and 50 °C, was defined as having a relative activity of 1. Conditions were: Controls: C-NL, no lipase; C-NW, not whipped; Whipped samples: W-A, speed setting 1 (136 rpm at agitator, 60 rpm at attachment) for 30 s (no foam formed); W-B, speed setting 3 (580 rpm at agitator, 255 rpm at attachment) for 60 s (foam visible); W-C, speed setting 3 for a further 2 min (3 min total). Bars with the same letter do not have a significant difference in lipase activity.

Table 2
Lipase activity, as methyl hexanoate formation, measured in intact milk fat globules (i.e., diluted cream) compared with globules disrupted by homogenisation at 30 MPa, as a function of temperature and incubation time.[a]

Incubation time	Incubation temperature	Absolute lipase activity		Relative lipase activity	Average relative lipase activity
		Intact MFG	Homogenised MFG		
Short	10 °C	46,692	423,813	0.11	
Medium	10 °C	76,118	746,935	0.10	
Long	10 °C	112,152	1,055,660	0.11	0.11[b]
Short	20 °C	243,150	756,945	0.32	
Medium	20 °C	608,015	1,701,200	0.36	
Long	20 °C	866,615	2,231,995	0.39	0.36[a]
Short	40 °C	125,690	2,433,140	0.05	
Medium	40 °C	341,990	5,794,390	0.06	
Long	40 °C	424,170	6,665,700	0.06	0.06[b]

[a] For absolute lipase activity, measurements of methyl hexanoate peak area were made under the different conditions tabulated where incubation time was the wait in the analysis queue. Relative lipase activity is lipase activity for intact MFG divided by lipase activity for homogenised MFG; Average lipase activity values with the same superscript letter were not significantly different.

esters because the trace amounts of esters formed at low temperature were readily measurable. However, temperature was still considered a critical variable to investigate due to possible effects on interfacial structure. The objective of this section was to investigate the effects of temperature on enzyme behaviour as determined by enzyme kinetics and interfacial properties and therefore demonstrate that the approach can be used at any temperature of interest (if microbial growth is controlled and enzyme is not denatured).

Increasing the assay temperature increased the lipase activity of the positive control (Table 2, homogenised MFG) in a manner consistent with Arrhenius kinetics (Van Boekel, 2008). Thus, for positive controls the enzyme appears to have ready access to substrate (interfacial fat), and temperature-dependent turnover of the enzyme determines amounts of esters formed.

In contrast, for samples containing native MFG, Arrhenius kinetics are not observed (Table 2, intact MFG). The enzyme appears to have minimal access to substrate and slow turnover at 10 °C. The enzyme access to substrate at 40 °C also appears minimal, but turnover is faster (as expected). At 20 °C the enzyme turnover is expected to fall between the two extreme conditions (as for the positive control), but the amount of product formed does not follow the expected trend, thus suggesting that at 20 °C the enzyme has significantly more access to substrate than at the other two temperatures.

Effectively the positive control (Table 2, homogenised MFG) is used to measure the enzyme turn-over (i.e., rate at substrate saturation) at each of the temperatures investigated (which characterises the enzyme kinetics), while the sample measurement (Table 2, intact MFG) is determined by the turnover and substrate concentration (which characterises the interface). Therefore, a comparison of the two measurements (Table 2, relative lipase activity) should characterise the interface in a manner that is independent of temperature effects on enzyme kinetics and only reflects temperature effects on substrate availability.

The observations in this experiment were generally consistent with prior literature on the effects of temperature on MFG and lipolysis (Deeth, 2006). That is, raw milk at collection (i.e., close to 40 °C evaluated in this experiment) was not susceptible to lipolysis, but cooling (spontaneous lipolysis) or cooling and rewarming (temperature-induced lipolysis) increased susceptibility to lipolysis (Deeth, 2006). Such reports from the literature represent confounded effects of enzyme kinetics (includes activators and inhibitors) and substrate availability. However, changes in interfacial structure and interfacial tension due to temperature changes in emulsions are well known (Rousseau, 2000) and are expected to affect access of lipases to interfaces. Clearly this is a subject that warrants further investigation and the assay developed here could be a useful tool to do so as measurements were readily made at temperatures between 10 and 40 °C.

3.6. *Comparison of lipoprotein lipase to lipase from* Pseudomonas cepacia

Principles presented thus far were not expected to be unique to LPL. Indeed, other authors have also found other lipases that readily discriminate emulsion samples based on the nature of globule interfaces (Deeth & Fitz-gerald, 1978; Mun et al., 2007). However, to further validate this work, and to start building an understanding of suitability of different lipases for probing MFGM a *Pseudomonas* lipase was assessed.

The behaviour of *P. cepacia* lipase in comparison with LPL is shown in Fig. 4. The results for Fig. 4 for LPL were consistent with those shown in Fig. 2, including a variation related to the incubation time as discussed in Section 3.3. The lipase from *P. cepacia* has significantly different behaviour in that this lipase comes closer to maximum enzyme turn-over (i.e., no accessible-substrate limitation) with the application of minimal homogenisation; this

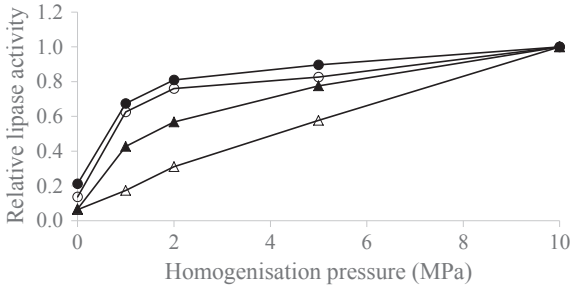

Fig. 4. Relative lipase activity measured in diluted cream (4% fat) after milk fat globules were disrupted by sample homogenisation at different pressures where lipase was either lipoprotein lipase (△, ▲) or lipase from *Pseudomonas cepacia* (○, ●). For each lipase results are from after a short (hollow symbols) or long incubation time (solid symbols). A sample homogenised at 30 MPa, and 50 °C, was defined as having a relative activity of 1.

observation is consistent with reports by others (Deeth, 2006). *Pseudomonas* lipases probably have surface properties that allow them to displace MFGM more readily than LPL can (Danthine & Blecker, 2014).

While the *Pseudomonas* lipase is more limited by enzyme turnover, in all but unhomogenised samples MFGM clearly remains a barrier to this lipase if MFG are not disrupted. That is, only the slightest change to MFG caused a very large change in the activity of *Pseudomonas* lipase. Therefore, the *Pseudomonas* lipase will be a more sensitive probe for changes to MFGM.

Bacterial lipases are often considered the cause of high FFAs in raw milk because they are not restricted by MFGM (Deeth, 2006); however, the *Pseudomonas* lipase was closer to LPL in behaviour than was expected. It is possible that this bacterial lipase was not representative of bacterial lipases in general. Alternatively, the differences between lipases may not be as large as the literature implies, possibly because the ability of lipases to access MFG is often measured under more extreme conditions (higher temperatures, much higher enzyme concentrations).

3.7. Effect of sample matrix on relative lipase activity measurements

In all prior sections, the experiments described were performed on diluted cream as a source of MFG in a simple matrix (~4% fat, ~0.2% protein and ~0.3% lactose). However, it is expected that there will also be a need to characterise interfaces in more complex matrices such as whole milk (~4% fat, ~3% protein and ~3% lactose). Therefore, samples of unhomogenised whole milk were analysed in parallel to samples of diluted cream, with both materials being analysed before and after whipping under the same conditions; results are presented in Table 3.

To compare different materials, each sample was spiked with homogenised cream to give a positive control.

In both whole milk and diluted cream the effect of whipping on MFG was detectable, despite the difference in matrix, as a change in absolute lipase activity of the samples (Table 3). Changes were also apparent in lipase activities expressed relative to the positive control. Thus, approaches developed here can probably be applied to samples more complex than diluted cream if the same principles are followed. In the future a more in-depth study is needed to explain how MFG disruption is influence by different matrices.

Table 3
Lipase activity, as methyl hexanoate formation, measured in samples of different types either as is or after addition of a spike of homogenised material, as a function of incubation time.[a]

Incubation time	Sample	Absolute lipase activity		Relative lipase activity
		Sample	Spiked sample	
Short	Diluted cream	15,395	184,636	0.08
Long	Diluted cream	82,003	1,189,602	0.07
Short	Whipped diluted cream	173,484	1,422,450	0.12
Long	Whipped diluted cream	410,547	2,485,072	0.17
Short	Whole milk	53,873	351,819	0.15
Long	Whole milk	70,050	561,216	0.12
Short	Whipped whole milk	284,381	785,610	0.36
Long	Whipped whole milk	374,174	1,145,397	0.33

[a] For absolute lipase activity, measurements of methyl hexanoate peak area were made under the different conditions tabulated where incubation time was the wait in the analysis queue. Relative lipase activity is lipase activity for the sample as is divided by lipase activity for the spiked sample.

4. Conclusions

The response of the lipase assay based on methyl ester formation was proportional to the concentration of disrupted MFG (i.e., diluted cream after homogenisation). Therefore, lipase activity of unknown samples could be compared with an appropriate disrupted MFG standard (internal or external) to determine a relative lipase activity that was proportional to the concentration of disrupted MFG even when assay conditions changed (e.g., different incubation times or temperatures).

Because assay responses were calibrated against standards for the material being measured (disrupted MFG), concentrations of esters were not measured. The advantages of such an approach for creating simple robust measurements were demonstrated. However, a disadvantage was the lack of an internationally accepted standard for disrupted MFG or MFGM, necessitating use of a standard that was manufactured in-house. The ability to reproducibly create emulsion standards, such as that developed in-house, with consistent and known accessibility to lipases should be investigated if this approach is to be widely adopted. Ideally, a stable reference material could be created and shared internationally. In the absence of an accepted standard, relative comparisons of samples using this approach still appear competitive with many alternative proposed approaches (Evers, 2004b).

The principle of using lipase activity as a probe of MFGM (or interfacial) status was not new (Evers, 2004b); however, the implementation of the test principle was often poor due to constraints created by lipase assays. Use of the more sensitive assay described here has removed many of these constraints, e.g., allowing samples to be measured at a more relevant temperature of 10 °C, while also permitting measurements at higher temperatures. Also, the assay conditions described here were significantly less perturbing of samples than those that have been used previously. Therefore, overall results were consistent with past reports while revealing an extra degree of detail, e.g., a detection limit for only 1% homogenised MFG in a blend. Furthermore, assay throughput was significantly increased because an incubation time equal to batch analysis queue times was acceptable.

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References

Andrewes, P. (2018). Indirect detection of lipase in UHT milk by measuring methyl ester formation. *International Dairy Journal, 79*, 1–4.
Beer, H. D., Wohlfahrt, G., McCarthy, J. E. G., Schomburg, D., & Schmid, R. D. (1996). Analysis of the catalytic mechanism of a fungal lipase using computer-aided design and structural mutants. *Protein Engineering Design and Selection, 9*, 507–517.
Danthine, S., & Blecker, C. (2014). Interactions of lipases with milk fat globule membrane monolayers using a Langmuir film balance. *International Dairy Journal, 35*, 81–87.
Deeth, H. C. (2006). Lipoprotein lipase and lipolysis in milk. *International Dairy Journal, 16*, 555–562.
Deeth, H. C., & Fitz-gerald, C. H. (1977). Some factors involved in milk lipase activation by agitation. *Journal of Dairy Research, 44*, 569–583.
Deeth, H. C., & Fitz-gerald, C. H. (1978). Effects of mechanical agitation of raw milk on the milk-fat globule in relation to the level of induced lipolysis. *Journal of Dairy Research, 45*, 373–380.
Evers, J. M. (2004a). The milkfat globule membrane—compositional and structural changes post secretion by the mammary secretory cell. *International Dairy Journal, 14*, 661–674.
Evers, J. M. (2004b). The milkfat globule membrane—methodologies for measuring milkfat globule (membrane) damage. *International Dairy Journal, 14*, 747–760.
Iametti, S. O., Versuraro, L., Tragna, S., Giangiacomo, R., & Bonomi, F. (1997). *International Dairy Journal, 7*, 375–380.

Magnusson, B., & Örnemark, U. (2014). *Eurachem guide: The fitness for purpose of analytical methods – A laboratory guide to method validation and related topics* (2nd ed.) Available from: www.eurachem.org.
Mun, S., Decker, E. A., & McClements, J. D. (2007). Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. *Food Research International, 40*, 770–781.
Rousseau, D. (2000). Fat crystals and emulsion stability — a review. *Food Research International, 33*, 3–14.
Singh, H., & Gallier, S. (2017). Nature's complex emulsion: The fat globules of milk. *Food Hydrocolloids, 68*, 81–89.
Van Boekel, M. A. J. S. (2008). Kinetic modelling of food quality: A critical review. *Comprehensive Reviews in Food Science and Food Safety, 7*, 144–158.
Verger, R. (1997). Interfacial activation of lipases: Facts and artefacts. *Trends in Biotechnology, 15*, 32–38.