Doctoral Dissertation

Effect of different chemically modified starches on the physico-chemical properties and microstructure of stirred non-fat yoghurt

Major: Bioresources Science

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Table of Contents

List of Tables

List of Figures

Abstract

The use of chemically modified starches for the improvement of physico-chemical properties and microstructure of non-fat stirred yoghurt was investigated. Yoghurts were prepared from non-fat and/or full-fat milk powder, sucrose and water. Three types of starches were added to the non-fat yoghurts after fermentation at 1% (w/w). They included: tapioca starch acetates (TSA-1, TSA-2, TSA-3), tapioca distarch phosphates (TDP-1, TDP-2, TDP-3) and native tapioca starch; with the modified starches varying in degree of substitution. Characterization of the starches included measurement of acetyl and phosphorus content, degree of substitution, swelling power, molecular weight distribution and RVA analysis for pasting properties. Yoghurt characterization included measurement of chemical properties, syneresis, particle size distribution and viscoelastic properties of the yoghurts were measured and the Herschel–Bulkley model used to describe their flow behaviour. Furthermore, interactions between milk proteins and modified starches attributed to protein surface hydrophobicity were characterized. Confocal laser scanning microscopy (CLSM) studies were also conducted to observe the microstructure of the yoghurt.

Analysis of the starches showed that modification had a significant influence on physico-chemical properties of the starches. The degree of substitution of tapioca starch acetates (TSA-1, TSA-2 and TSA-3) was found to be 0.019, 0.026 and 0.068, respectively. As for the tapioca distarch phosphates (TDP-1, TDP-2 and TDP-3), the degree of substitution was 0.0058, 0.0063 and 0.0081, respectively. Acetylation increased the swelling power and peak viscosity of the tapioca starch, reducing its pasting temperature and reducing the tendency of retrogradation. Cross-linking starch increased pasting temperature and peak viscosity but reduced the swelling power and increased the tendency of retrogradation of the tapioca starch. These characteristics would have an impact on the final yoghurt product. Results showed that yoghurts with starch acetates exhibited higher yield stress, consistency coefficient (K) values, hysteresis loop area,

storage modulus (G') and loss modulus (G''). Protein surface hydrophobicity was significantly influenced by the addition of starch acetates and TSA-3 yoghurt exhibited the lowest values. This is due to greater interaction between starch chains and casein micelles and the increased swelling of these starches in the serum phase.

In the microstructure evaluation, all non-fat yoghurts with starch were characterised by having densely packed protein particles in the form of large aggregates surrounded by an aqueous region and with fewer connections between the aggregates. Starch acetate-added yoghurts had a higher number of aggregates as well as less porosity in the casein network when compared to the native and distarch phosphate-added yoghurts. This study concluded that the addition of TSA-3 starch is the most suitable stabilizer in non-fat stirred yoghurt. It had the lowest syneresis among the non-fat yoghurts and could stabilize the protein network as a result of interaction between the milk proteins and starch chains.

Chapter 1

Literature review

1.1. Definition, classification and composition of yoghurt

Yoghurt is a product obtained from slow lactic fermentation of milk by a mixed starter culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* (Tamime & Robinson, 2007). However, in some countries, other suitable lactic acid bacteria such as *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *lactis* are permitted for use as starter cultures (Tamime, 2002). *Leuconostoc* and *Lactococcus* strains are often incorporated as adjunct cultures to enhance the flavour of yoghurt. Furthermore, probiotic strains belonging to the genera *Lactobacillus* (e.g. *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. reuteri*, *L. plantarum*) and *Bifidobacterium* (e.g. *B. longum*, *B. bifidum*, *B. breve and B. infantis*) are added for their proposed health benefits (Chen *et al*., 2017; Mckinley, 2005).

Yoghurts can be categorised based on their chemical composition, their method of production, their f1avour and the nature of post-incubation processing as shown in Table 1 (Tamime & Deeth, 1980). Industrially, yoghurts can be largely divided into two types: set-type and stirred-type. In set yoghurt manufacture, the yoghurt is made in retail containers producing a continuous undisturbed gel structure while in stirred yoghurt, the gel is disrupted by stirring (agitation) and then it is packaged. Stirred yoghurts should have a smooth and viscous texture (Tamime & Robinson, 1999).

Table 1. Classification of yoghurts

Basis of classification	Types of yoghurt
1. Chemical composition	Full, low/reduced or non-fat yoghurt
2. Method of production and physical structure of the coagulum	Set or stirred or drinking yoghurt
3. Flavouring of yoghurt	Plain/natural, fruit and flavoured yoghurt
4. Post-incubation processing	Pasteurized/UHT yoghurt, concentrated yoghurt, frozen yoghurt and dried yoghurt

Yoghurt, similar to milk, is an excellent source of protein, calcium, phosphorus, riboflavin, thiamin, vitamin B12, folate, niacin, magnesium and zinc. Since lactose in milk is converted into lactic acid during fermentation, lactose-intolerant people can consume yoghurt without any adverse effect. In addition, yoghurt consumption causes a small increase in stomach pH and this reduces the risk of the pathogen passage and the effects of low gastric juice secretion problem (Nguyen & Hwang, 2016).

1.2. Manufacture of yoghurt

Manufacture of yoghurt consists of three basic steps: preparation and heat treatment of milk, incubation and cooling and packaging process (Benezech & Maingonnat, 1994).

1.2.1. Preparation and heat treatment of milk

Cow's milk is generally used as the raw material for yoghurt production, although the milk from goat, sheep, camel and buffalo is equally suitable for fermentation (Nguyen *et al*., 2018; Jumah *et al*., 2001). Jumah *et al*. (2001) reported that the milk source greatly impacted the rheological properties of yoghurt. Sheep milk had the highest viscosity, followed by goat, cow and camel milk. They attributed this to variation in the chemical composition of milk, namely total solids and protein content. Depending on the product (i.e. full-fat, low-fat or non-fat), if it

contains fat, the milk is homogenized at 10-20 MPa to prevent creaming during fermentation (Fox *et al*., 2015). Additionally, Tamime (2002) reported that homogenization causes an increase in the whiteness of the product, increase in viscosity of the product due to interaction and/or adsorption of the fat globules onto the casein micelles and reduced why separation due to increase in its hydrophilicity and water-holding capacity as a result of the interaction of the proteins.

Heating of milk is an essential step in the processing of yoghurt. The main reason for heat treatment of milk is to improve its keeping quality by reducing the number of living microorganisms. In addition, heat treatment results in denaturation of whey proteins which either associate with the casein micelle or form soluble whey protein aggregates to achieve desirable properties in the final product (Loveday *et al.*, 2013). Temperature-time profile ranging from 80-85°C for 30 min to 90-95°C for 5 min is considered to be adequate for producing high quality yoghurts (Soukoulis *et al*. 2007). Yoghurt prepared with unheated or inadequately heat-treated milk is characterized by having poor texture, weak firmness and increased whey separation (Tamime & Robinson, 1999).

1.2.2. Incubation

The choice of the type of strain used acts mainly as an improving factor in the viscosity. Exopolysaccharide (EPS)-producing cultures have been used to improve the texture of yoghurt. De Vuyst *et al*. (2001) reported that strains of both *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* can produce exopolysaccharides that improve yoghurt viscosity, enhance texture, mouthfeel and water retention capacity.

For optimum incubation, the temperature is closely related to the type of strains used of fermented milk. The fermentation process involves the inoculation of pasteurized milk with concentrated cultures of bacteria; the milk is then incubated at 40-44°C for 4-5 h. During fermentation, these bacteria convert lactose into lactic acid or other metabolites (glycolysis),

hydrolyze caseins into peptides and free amino acids (proteolysis) and breakdown milk fat into free fatty acids (lipolysis) (Chen *et al*., 2017). The reduction in pH, due to the production of lactic acid, destabilizes casein micelles and coagulation occurring around pH 4.6. The other metabolic activities of lactic acid bacteria are responsible for the coagulation of milk proteins and the production of various compounds that impart the organoleptic and textural characteristics of the final product. Lactic acid had been found as a key taste component in yoghurt, as well as carbonyl compounds such as acetaldehyde, diacetyl, acetoin and 2-butanone. These volatile compounds are responsible for the characteristic aroma of plain yoghurt (Tamime & Robinson, 1999).

1.2.3. Cooling and packaging

The coagulated milk is then cooled quickly to 4 - 10°C to slow down the fermentation process, after reaching the desired final pH. For stirred yoghurts, the yoghurt gel is mixed at the end of fermentation, then cooled and packaged. In industry, the gel is first cooled from the incubation temperature to approximately 20°C before filling in retail containers and then further cooling at 4°C. Renan *et al*. (2009) reported that filling at this temperature could prevent excessive structural breakdown. During stirring the viscosity of the yoghurt decreases but increases again during cold storage. This structure recovery or rebodying is as a result of the decrease in temperature, over-acidification and the production of exopolysaccharides (EPS) by bacteria during storage.

1.3. Formation of milk coagulum

The basic building blocks of yoghurt are the casein micelles. Caseins constitute approximately 80% of the protein in bovine milk, with four main types (α_{s1}-, α_{s2}, β-, and κ-CN) in combination with micellar or colloidal calcium phosphate nanoclusters in the form of aggregates called casein micelles (Lucey, 2002). Schmidt (1982) observed electron microscopic images of the casein

micelle having a raspberry-like structure and proposed that the structure of casein micelles is divided into discrete subunits (submicelles) with distinctly different properties from an outside "hairy" layer of κ -casein.

Yoghurt production may be divided into primary (heat treatment) and secondary (acidification) stages. The main aggregates formed as a consequence of heat treatment of milk, are complexes formed by aggregation of denatured whey proteins and complexes between β-lactoglobulin and κ-casein on the surface of the casein micelles via disulphide bonds and hydrophobic interactions. Corredig and DaLgleish (1996) reported that at temperatures below 70°C, the interaction is mostly caused by hydrophobic interactions while at higher temperatures it is mostly caused by disulphide bonds.

During acidification, the extended portion of the κ-casein collapses, there is a decrease in charge repulsion and the micelles aggregate (Everett & McLeod, 2005). The change of micellar conformation and precipitation of milk protein are critical to the development of yoghurt. Lee and Lucey (2010) reported that aggregation of casein micelles is observed when the pH of the milk falls below 5.0 and the solubility of colloidal calcium phosphate of the milk increases. When the pH becomes close to the isoelectric point of casein (pH 4.6), there is a decrease in electrostatic repulsion is due to a decrease in the number of charged regions of κ-casein leading to a low net negative charge. Casein micelles and denatured whey protein interact to form chains and clusters through hydrophobic and electrostatic bonds leading to a gel structure.

1.4. Role of fat in yoghurt

The presence of fat in dairy products has a considerable impact on their physical properties, rheological and textural characteristics and microbiological stability (Brennan & Tudorica, 2008). Fat globules of homogenized milk are part of the gel network acting as structure promoters of protein network in yoghurt. They interact with each other and with denatured whey proteins associated with casein micelles in the serum during acidification. Furthermore, whey proteins

can adsorb onto the fat globule surface and enhance the interaction among themselves (Lucey *et al*., 1998). As a result, the higher number of fat globules could lead to the development of multiple interactions between fat globules, whey proteins and casein micelles that strengthens the 3D gel network (Aguilera & Kessler, 1989). In reduced-fat yoghurt, the number of fat globules is not enough to strengthen the gel network and the texture is determined by protein-protein interactions (Nguyen *et al*., 2017). Full-fat yoghurt, low-fat yoghurt and non-fat yoghurt contain at least 3.25% milkfat, between 0.5% and 2% milkfat and less than 0.5% milkfat, respectively, and each of these products contains at least 8.25% milk solids-not-fat before the addition of bulky flavours. These fat levels correspond to the Food and Drug Administration requirement for nutritional labelling of yoghurt, low-fat and non-fat yoghurt (Chandan, 1997).

Concerns related to low viscosity, poor texture and syneresis result from a modification of the structure of the gels (Figure 1). The partial or total removal of fat from yoghurt, also, decreases the overall quality perceived by the consumer (Cayot *et al*., 2008). To modify the texture perception, yoghurt can be modified by fortifying the milk with dairy-based ingredients, non-dairy ingredients or a combination of both before heat treatment and acidification (Oh *et al*., 2006; Sandoval-Castilla *et al*., 2004).

Figure 1. Photographs showing wheying-off and poor texture in a set-style (left) and stirred-style (right) non-fat yoghurt

1.5. Food stabilizers used the yoghurt manufacture

Food stabilizers have two basic functions in dairy products in terms of water binding and texture improvement. In this way, they increase viscosity and reduce syneresis. In addition, they positively impact yoghurt by enhancing appearance and mouthfeel (Shi *et al*., 2017).

1.5.1. Dairy-based ingredients

Skim milk powder (SMP) is commonly used, however, other dairy ingredients such as whey protein concentrates or isolate (WPC or WPI) and sodium (Na)-caseinate or calcium (Ca)-caseinate are also used to increase the total solids content of the yoghurt mix and improve gel firmness in reduced-fat yoghurts (Isleten & Karagul-Yuceer, 2006). Yoghurt firmness and the resistance against wheying off are improved as the protein content increases (Soukoulis *et al*. 2007). Dairy-based fat replacers have been used in low and non-fat yoghurts with varying degrees of success. However, fortification with these expensive dairy commodities affects production costs and their addition to yoghurts causes powder flavour in the final product (Nouri *et al*., 2011).

Mistry and Hassan (1992) investigated the effect of adding high milk protein powder manufactured from skim milk to non-fat yoghurts and described yoghurts with more than 5.6% protein as being too firm and had an astringent flavour. Whey protein concentrates are produced by ultrafiltration of whey to enrich the protein fraction by removal of lactose, minerals and other low molecular weight components. Sodini *et al*. (2005) found that yoghurts enriched with WPC possessed higher water-holding capacities than a control enriched with skim milk powder. They attributed this to concentrations of the various nitrogen fractions of the WPC determining the functional properties of WPC in yoghurts. Modler *et al*. (1983) investigated the effect of adding of three casein-based products and three whey-based ingredients on the texture of skim yoghurt and found that the casein-based yoghurts were firmer with less syneresis than yoghurts based on whey protein.

1.5.2. Non-dairy fat ingredients

Addition of food hydrocolloids such as gelatin, pectin and various starch products.to yoghurt is a practical solution for increasing the viscosity and decreasing the syneresis. Generally, they function by binding water, reacting with the milk constituents (mainly proteins) and stabilize the protein network preventing free movement of water (Tamime & Robinson, 1999).

1) Gelatin

Gelatin is one of the widely used hydrocolloids and ranks second only to starch in terms of volume and value (Wanous, 2004). It is a protein ingredient derived from skins, bones and connective tissues of animals via partial denaturation and hydrolysis of native collagen extracted (Shi *et al*., 2017). Gelatins are chiefly produced from bovine and porcine sources, but they might also be extracted from fish and poultry. Gelatin improves the texture of low-fat yoghurts, resulting in a firmer gel with reduced syneresis. Studies done by Ares *et al*. (2007) showed that the presence of hydrocolloids could alter these parameters. They found that low-fat yoghurts with 6 mg/g of gelatin did not show syneresis and showed the highest sensory viscosity, creaminess and mouth-feel. This effect has been attributed to gelatin interactions with casein micelles in yoghurt, developing 3D networks, which subsequently prevents the serum separation of yoghurts. Fiszman *et al*. (1999) had previously investigated the effect of gelatin addition on the microstructure of acidified milk gels and yoghurt and found that gelatin retained the aqueous phase and reduced syneresis efficiently. Gelatin interacted with casein network forming flat sheets or surfaces with or enclosing casein granules in several zones which confers yoghurt with a uniform microstructure.

2) Pectin

Pectin is widely used as a functional ingredient in the food industry due to its ability to form aqueous gels and has been used in jams and jellies, fruit preparations, fruit drink concentrates,

fruit juice, desserts and fermented dairy products. Pectin is a prevalent hydrocolloid utilized in acidified milk as high methoxyl pectin (HMP) and low methoxyl pectin (LMP). It has been observed that pectin adsorbs on caseins at the beginning of acidification, affecting the conformation of casein micelles at the pH range of 5.0–5.8 (Nakamura *et al*., 2006). The stability is enhanced due to strong steric repulsions generated between pectin chains. This is induced by both depletion flocculation and electrostatic attraction between the polysaccharide and casein resulting in decreased viscosity and increased water holding capacity.

3) Starch

Starch is a polysaccharide made up of glucose units which are linked together via glycosidic linkages and is composed of amylose and amylopectin. Amylose is a linear polysaccharide with α-1,4-linked D-glucopyranose molecules. Amylopectin is a highly branched polysaccharide with α-1,4-linked D-glucose backbones and exhibits about 5% of α-1,6-linked branches (Charoenthai *et al*. 2018; Thomas & Atwell, 1999). Starch, including native and particularly modified starch, accounts for more than 85% of all hydrocolloids used in food worldwide (Wanous, 2004).

Starches meet the functional properties required in food products such as thickening and stabilization, gelling, bulking and play as water retention agent. During the heating process, the starch granules found in the serum swell. Starch swelling is due to the expansion of amylopectin; disruption of the crystalline region is caused by an expansion of the amorphous region, leading to enhancement of the interaction of starch molecular chains with water. Amylose is leached from starch granules to water during heating and the leached amylose interacts with molecular chains in the amylopectin of swollen starch granules, forming a 3D network (Kurakake *et al*. 2009). The swollen starch is converted to a rigid structure by a decrease in the temperature as a result of rearrangement of amylose (retrogradation).

Starch is a cost-effective thickening agent widely used in yoghurt manufacturing to increase viscosity, improve mouthfeel and reduce syneresis (Ares *et al*., 2007). However, native starch is insoluble in water and it easily retrogrades and loses viscosity when subjected to heat treatment, making modification necessary to overcome these shortcomings. Hence starch modification improves its functional characteristics, it might be used in many specific food applications. Starch modification is carried out by changing its molecular structure and can be done chemically, physically or their combinations.

Physical modification of starch is mainly accomplished by heating and/or mechanical shearing that changes the granular structure and converts native starch into cold water-soluble starch or small-crystalline starch (Thomas & Atwell, 1999). Some physical methods are gelatinisation, thermal inhibition, osmotic-pressure treatment, glow discharge plasma treatment, ultra-high pressure treatment, freezing, retrogradation, annealing and heat moisture treatment.

Another technique involved in enhancing starch properties is chemical modification that involves treatment of starch with chemical reagents to attach new chemical substituent groups, effect molecular scission, promote oxidation or molecular rearrangements (Wurzburg, 1986). Chemical modification causes marked changes in physico-chemical properties of starch due to the introduction of functional groups into the starch molecules as shown in Table 2. It has been reported that addition of low levels of starch (up to 1%) on the properties of acid gels is additive, but higher levels (1.5–2.0%) produced a diminished effect on the storage modulus (Oh *et al*., 2007). The effect of native and chemically modified starches on yoghurts has been tested. Lobato-Calleros *et al*. (2014) reported that modified starch they used reduced syneresis and enhances rheological properties, yoghurt stability during storage increased with starch addition. Nguyen *et al*. (2017) found that low-fat yoghurt with 1% hydroxypropyl starch increased the thickness of the sample but also created other undesirable mouthfeel attributes, such as chalkiness and lumpiness. Pang *et al*. (2019) found that the degree of cross-linking and acetylation is crucial for the application of acetylated distarch phosphates in yoghurt. Cui *et al*.

(2014) had previously reported that cross-linked acetylated starch adsorbed onto the surface of the casein micelles strengthening the casein network. Radi *et al*. (2009) used acid-treated wheat starch and acid-treated cross-linked wheat starch in low and non-fat and found yoghurts with acid-treated starch were unacceptable to the consumers although both starches significantly reduced syneresis.

Table 2. Chemically modified starches used in the dairy industry and some of the attributes imparted by modification (BeMiller & Whistler, 2007; Singh *et al*., 2007; Thomas & Atwell, 1999).

Chemical modification	Types of starches	Attributes imparted after modification
1. Stabilization		
▪Etherification	▪Hydroxypropyl starches	▪Improved clarity of paste, higher viscosity, reduced syneresis and freeze-thaw tolerance
▪Esterification	▪Starch acetates, starch octenylsuccinates, monostarch phosphates	▪Higher paste clarity, lower gelatinization temperature and decreased setback of pastes
2. Cross-linking	Distarch phosphates, distarch adipates	Increased gelatinization and pasting temperatures, increased paste viscosity, higher stability of granules towards high shear and acidic conditions
3. Dual modification (Cross-linking and stabilization)	Hydroxypropylated distarch phosphates, phosphorylated distarch phosphates, acetylated distarch phosphates, acetylated distarch adipates	Stability against acid conditions, heat treatment and high shear and delayed retrogradation during storage
4. Conversion		
▪Oxidation/ bleaching	▪Oxidized starches, bleached starches	▪Low viscosity, high clarity and low temperature tolerance
▪Acid hydrolysis	▪Acid thinned starches	▪Decreased viscosity of pastes, lower gelatinization and pasting temperatures and increased solubility
▪Pyroconversion (dextrinization)	▪Pyrodextrins	▪Low to high solubility, low to high viscosity depending on reaction conditions
▪Enzyme hydrolysis	▪Maltodextrins, glucose, glucose syrups, high-fructose syrups	▪Low viscosity, sweetness, increased water-binding properties

Chapter 2

Effect of tapioca starch acetates and tapioca distarch phosphates on physico-chemical properties of non-fat yoghurt

2.1. Introduction

Yoghurt is made by lactic acid fermentation of fresh milk using starter cultures to give a pH value of 3.8-4.6 (Tamime, 2002). Low-fat or non-fat yoghurts are popular due to their nutritional characteristics. However, reducing the fat content of yoghurt alters its structural and mechanical characteristics, resulting in poor food texture characteristics and high syneresis (Pereira *et al*., 2006; Sandoval-Castilla *et al*., 2004). The addition of dairy-based ingredients, non-dairy ingredients or a combination of both increases the total solids content of the milk resulting in desirable low fat or non-fat yoghurts (Sandoval-Castilla *et al*., 2004). In the past, starches have proven useful for their role as gelling, stabilising and thickening agents in different food applications. However, in the dairy industry, native starches are not preferred since they possess low shear and thermal resistance and have a high tendency to retrogradation (Corredig *et al*., 2011). These shortcomings of native starches could be overcome by introducing functional groups into the molecules (Singh *et al*., 2007). In yoghurt-making, chemically modified starches can exert some positive effects (Bravo-Núñez *et al*., 2019; Cui *et al*., 2014; Pang *et al*., 2019; Sharma *et al*., 2018). During heat treatment of milk, whey proteins, particularly β-lactoglobulin, are denatured leading to the formation of soluble and micelle bound whey protein aggregates through hydrophobic interaction, electrostatic interaction and disulphide bonding (Krzeminski *et al*., 2011). These aggregates interact with casein micelles during acidification of milk. A decrease in pH leads to an increase in the attachment of whey proteins to the casein micelle. On

the other hand, starches gelatinize when heated in excess water resulting in disruption of the granular structure, swelling and hydration and solubilization of starch chains.

The combined effect of the swollen starch granules, milk proteins adsorbing onto starch chains and the changes in the structure of milk proteins during heating and acidification leads to the firm structure of the yoghurt (Noisuwan *et al*., 2011; Oh *et al*., 2007). A proposed schematic representation of the formation of stirred yoghurt gels from pasteurized milk to stirred yoghurt followed by the addition of modified starches to non-fat yoghurt is shown in Figure 2. The use of modified starch may increase yoghurt viscosity and strengthen the rigidity of the casein network by binding water and interact with other milk constituents, such as proteins thereby inhibiting syneresis. Although the addition of modified starches has been investigated in the processing of low-fat yoghurt, it is difficult to independently evaluate the effect of the starches on yoghurt properties because the addition of starch prior to fermentation also changes the final starch and total solids contents.

Therefore, the objective of this research was to analyse the syneresis, rheological properties and protein surface hydrophobicity of non-fat stirred yoghurts made using starch acetates and distarch phosphates at different levels of modification. The starch was added after acidification and before heat treatment of yoghurt.

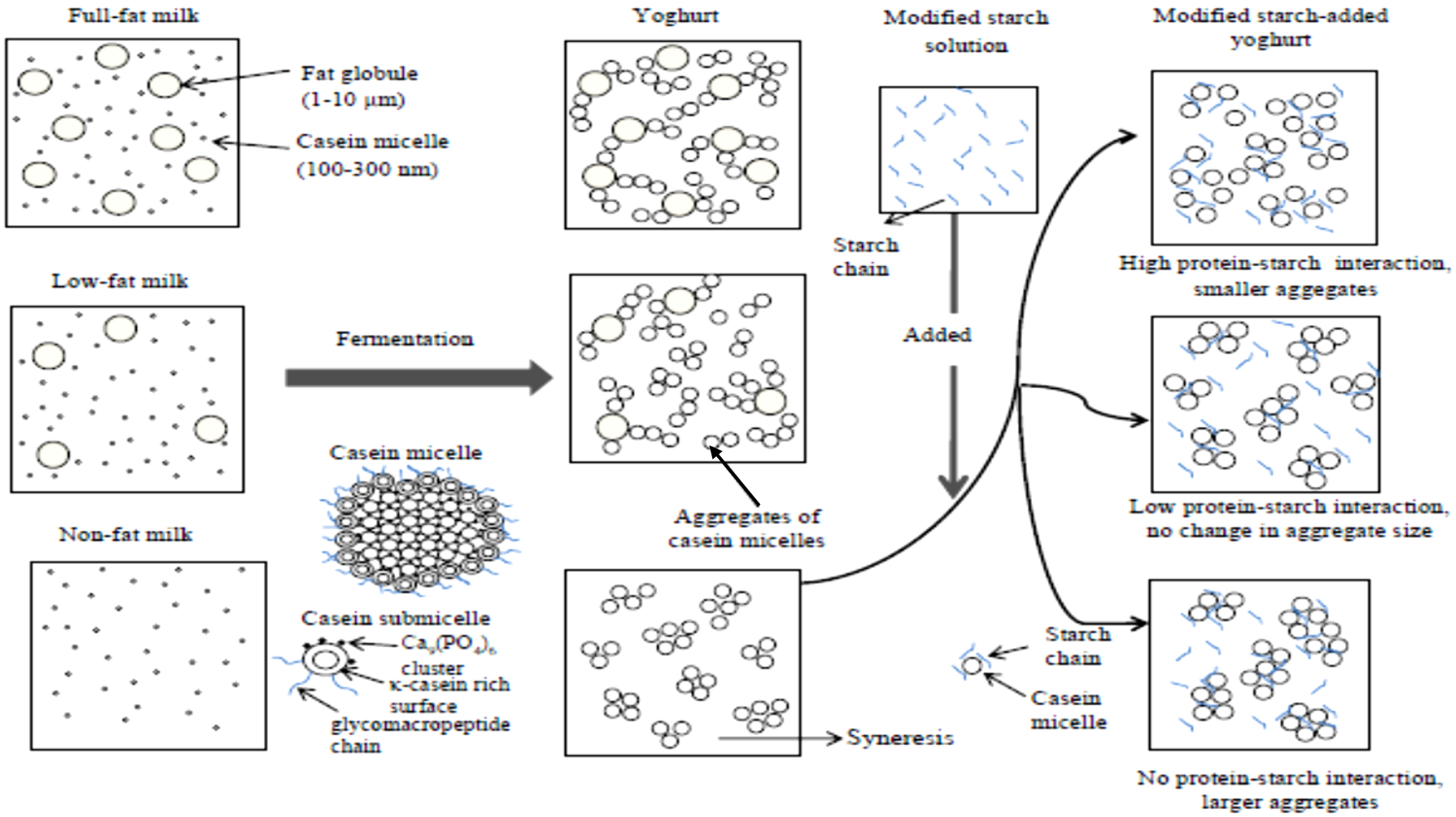


Figure 2. A schematic representation of the formation of stirred yoghurt gels from pasteurized milk to stirred yoghurt followed by the addition of modified starches

2.2. Materials and Methods

2.2.1. Materials

Three types of tapioca starch acetates with different degree of substitution (TSA-1, TSA-2 and TSA-3), three types of tapioca distarch phosphates with different degree of substitution (TDP-1, TDP-2 and TDP-3) and native tapioca starch were obtained from J-Oil Mills Inc., Tokyo, Japan. Low-heat-treated non-fat dry milk was obtained from Megmilk Snow Brand Co., Ltd, Tokyo, Japan. Freeze-dried non-ropy producing yoghurt culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (YC-X11 Yo-Flex®) was obtained from Chr. Hansen A/S, Denmark. 8-anilinonapthalene-1-sulfonic acid (MP Biomedicals LLC, Illkirch, France) was used as the fluorescent probe.

2.2.2. Physico-chemical properties of starches

1) Degree of substitution (DS) of starch acetates and distarch phosphates

The acetyl content and DS of starch acetate were determined according to Ando *et al*., (2013). Starch (5.0 g) was added to 50 mL of distilled water before adding a few drops of phenolphthalein indicator and then 0.45 M NaOH. After agitation for 30 min, the suspension was titrated with 0.5 M HCl. For the blank, starch was excluded. The content of acetyl and DS was calculated as follows:

$$Acetyl\ \% = \frac{(B - S) \times M \times 0.043}{W} \times 100 \quad (1)$$

where B is the volume of HCl required for blank titration, S is the volume of HCl required for sample titration, W is the weight (g, dwb) of acetylated starch sample and M is the molarity of HCl solution.

$$DS = \frac{162 \times Acetyl\ \%}{4{,}300 - (42 \times Acetyl\ \%)} \times 100 \quad (2)$$

where 162 is the molecular weight (Mw) of glucose unit, 4,300 is 100 × Mw of acetyl group, and 42 is the Mw of acetyl group minus the Mw of hydrogen atom.

For phosphorus content and DS of cross-linked starches, the phosphorus content was colorimetrically determined by reaction with ammonium molybdate-vanadate solution according to AOAC Method 986.24 (1995). The absorbance of the prepared sample was measured at 460 nm using a spectrophotometer at 562 nm (V560, JASCO Corp., Japan). A calibration curve was prepared with monobasic potassium phosphate solutions. Phosphorus contents (%P) were calculated as follows:

$$\%P = \frac{a \times 2000}{V \times W} \quad (3)$$

where *a* is the amount of phosphorus in the sample solution read from the calibration curve (mg/mL), *V* is sample aliquot used and *W* is the weight of sample (g, dwb).

Degree of substitution in cross-linked starches was calculated using the equation given by Wongsagonsup *et al*. (2005).

$$DS = \frac{324 \times \%\ P}{3{,}100 - (96 \times \%P)} \times 100 \quad (4)$$

where 162 is the Mw of glucose unit, 3,100 is 100 × Mw of phosphorus, and 96 is the Mw of monophosphate substituent group minus the Mw of hydrogen atom.

2) Swelling power

Starch (2.50 g) was dispersed in 100 ml deionized water and heated at 95°C for 30 min, followed by centrifugation at 1,500×*g* for 30 min. The supernatant was decanted and the weight of the wet residue noted. Swelling power was determined as the ratio of the weight of swollen starch granules after centrifugation to their dry weight.

3) Molecular weight distribution

The molecular weight distribution of starch was determined using the high performance size exclusion chromatography (HPSEC) system. Starch samples (0.1% w/v) were dissolved in dimethyl sulfoxide (DMSO) and injected into an HPSEC system (JASCO Corp., Japan) equipped with a refractive index detector. One TSKgel guard column (6.0 mm I.D. × 400 mm, particle size 13 μm) and one TSKgel α-M (7.8 mm I.D. × 300 mm, particle size 13μm)(Tosoh Bioscience, Japan) were used. The columns were eluted with 5 mM sodium nitrate in DMSO at a flow rate of 0.3 ml/ min maintained at 40°C. The system was calibrated with pullulan standards (Sigma-Aldrich Production, St Louis, USA) with molecular weights ranging from 6,200 to 805,000. The retention time at the maximum height of each peak was taken to represent the retention time for that particular molecular weight and molecular weight distribution (%) was calculated.

4) RVA analysis

The pasting properties were determined using Rapid Visco Analyser (RVA-4, Newport Scientific, Australia). Starch (3.0 g, 14% moisture basis) was dispersed in 25.0 g of deionised water. The suspension underwent a controlled heating and cooling cycle under constant agitation (160 rpm) where it was held at 50°C for 3 min, heated from 50 to 95°C at 6°C/min and held at 95°C for 7 min, cooled to 40°C at 6°C/min and held at 40°C for 7 min. Pasting temperature, peak viscosity, trough viscosity, breakdown viscosity and setback viscosity were recorded.

2.2.3. Yoghurt production

Reconstituted milk was prepared by dissolving skim milk powder (12% (w/w)) and sucrose (6% (w/w)) in deionized water. Full fat yoghurt was prepared with full-fat milk powder and low-fat yoghurt was prepared with skim milk and full-fat milk powder. Milk samples were heated to 90ºC for 5 min and then cooled to 43ºC before inoculation with 0.002% (w/w) yoghurt starter. Fermentation was carried out in a water bath (NCB-3100, Tokyo Rikakikai Co., Ltd, Japan) at 43°C for 5 h. Starch solutions (1% w/w) were added after which the yoghurts were heated to 72°C for 10 min to stop the fermentation process. The yoghurts were then cooled to 5°C for further analysis.

2.2.4. Physico-chemical properties of yoghurt

1) Titratable acidity

Yoghurt (10.0 g) was mixed with deionised water (30 mL) and titrated with 0.1 N NaOH. Phenolphthalein (1 mL) was added before titration as an indicator to an endpoint of faint pink colour. The results were expressed as a percentage of lactic acid.

$$Titratable\ acidity\ (\%) = \frac{90 \times V \times N}{W \times 1000} \times 100 \quad (5)$$

where 90 is molecular weight of lactic acid (g/mol), V is the volume of NaOH used (mL), N is normality of NaOH (mol/L) and W is the sample weight (g).

2) Protein content determination

The protein contents of the yoghurt were obtained using the Kjeldhal method for nitrogen and was converted to the equivalent protein by a numerical factor of 6.38.

3) Fat content determination

The fat of the yoghurt samples was extracted according to the Roese-Gottlieb method (IDF, 1987). This method determines milk fat by ether extraction followed by solvent evaporation. The fat content was calculated as:

$$\text{Fat}\ (\%) = \frac{\text{Weight of fat}}{\text{Weight of yoghurt}} \times 100 \quad (6)$$

4) Syneresis

Yoghurt (20.0 g) was centrifuged at 100×g for 15 min at 4°C. The clear supernatant was poured off, weighed and expressed as per cent weight relative to the original weight of yoghurt.

5) Particle size analysis

The particle size distribution of the samples was obtained using a laser diffraction particle size analyzer (LS230, Small-Volume Mode, Beckmann Coulter, USA). Yoghurt samples were dispersed in deionized water and measurements of particle size were obtained at an obscuration of 14–15% and polarization intensity differential scattering (PIDS) of 45–55%.

6) Protein surface hydrophobicity

The protein surface hydrophobicity of the samples was determined according to Bonomi *et. al*. (1988). The relative fluorescence of the samples was measured using a fluorescence spectrometer (FP-8600, Jasco Corp., Japan) at room temperature (20–24°C) with 8-anilinonapthalene-1-sulfonic acid (ANS) as the fluorescent probe. The excitation wavelength (λ_{ex}) was set to 390 nm and the emission wavelength (λ_{em}) was set to 480 nm. Yoghurt samples diluted with 50 mM phosphate buffer (pH 6.8) were then titrated with increasing concentration

of ANS solutions until no further increase in fluorescence was observed. Samples without ANS were measured as blank.

From ANS curves, protein surface hydrophobicity (PSH) was calculated as:

$$PSH = \left[\frac{F_{max}}{K_d \times P}\right] \quad (7)$$

where F_{max} is maximum fluorescence, K_d is the ANS concentration required to obtain half the value of F_{max} and P is protein content.

7) Flow behaviour

Flow behaviour tests were carried out using a dynamic rheometer (ARES-G2, TA Instruments, USA) with the cone and plate geometry measuring system (ø 25 mm, cone angle 1°) at 20°C. Flow curves were obtained with a range of shear rate from 0.1 to 200 s^{-1} for 90 s (2.2 s^{-1}/s, rising curve) and 200 to 0.1 s^{-1} for 90 s (descendent curve) at 20°C and the shear stress values were then recorded. The flow behaviour was determined using the Herschel–Bulkley model.

$$\sigma = \sigma_0 + K \cdot \dot{\gamma}^n \quad (8)$$

where σ is shear stress (Pa), σ_0 is the yield stress (Pa), K is the consistency index (Pa·s^n), $\dot{\gamma}$ is shear the rate (s^{-1}) and n is flow behaviour index which indicates the closeness to Newtonian flow (n<1 indicates shear-thinning liquid). The hysteresis loop area, which is the area between the upward and the downward curve of shear stress against shear rate, was also calculated. The thixotropic index was calculated as the ratio between the hysteresis loop area and the area under the up curve.

8) Dynamic viscoelasticity testing

Dynamic viscoelasticity tests were carried out using a dynamic rheometer (ARES-G2, TA Instruments, USA) with the cone and plate geometry measuring system (ø 25 mm, cone angle 1°) at 20°C. Amplitude sweep tests were performed using a strain sweep of 0.001-2.5 (6.28 rad/s) at 20°C to determine the linear viscoelastic range for the yoghurt samples. Frequency sweeps were carried out under an angular frequency (ω) range of 0.1 rad/s to 100 rad/s (0.005 strain). The storage modulus, G', the loss modulus, G'' and the mechanical loss tangent ($\tan \delta = G''/G'$) were obtained as a function of ω.

2.2.5. Statistical analysis

IBM *SPSS* Statistics 21 software (IBM Corp., USA) was used to perform statistical analyses. Analysis of variance (ANOVA) and Tukey's HSD test were performed to determine significance at $P < 0.05$.

2.3. Results and Discussion

2.3.1. Physico-chemical properties of starches

1) Degree of substitution (DS) of starch acetates and distarch phosphates

Acetylation degrees of the acetylated starches (TSA-1, TSA-2 and TSA-3) are shown in Table 3. The degree of substitution of tapioca starch acetates (TSA-1, TSA-2 and TSA-3) was found to be 0.019, 0.026 and 0.068, respectively. TSA-3 had significantly higher ($P < 0.05$) acetyl content and DS indicating that it had a higher number of acetyl groups incorporated into the starch chain. The use of acetylated starches is completely dependent on the degree of substitution. The introduction of acetyl groups interrupts the ordered structure of native starch and interferes with the reassociation of amylose and amylopectin molecules in the gelatinized starch, leading to a decrease in the gelatinization temperature, an increase or decrease in the swelling power and solubility along with the storage stability. These characteristics the degree of substitution (DS) and the percentage of acetyl groups determine the use of starch acetate, for example, for food application; the Food and Drug Administration recommends a percentage of acetyl groups of less than 2.5 g/100g (López *et al*., 2010). Differences in DS and acetyl content can be attributed to the type of reagent, concentration, pH, presence of a catalyst, reaction time, botanical origin and characteristics of size and structure of starch granules (Colussi *et al*., 2015).

The level of cross-linking of the distarch phosphates (TDP-1, TDP-2 and TDP-3) are shown in Table 4. The degree of substitution for the tapioca distarch phosphates (TDP-1, TDP-2 and TDP-3) was 0.0058, 0.0063 and 0.0081, respectively. Results show that TDP-3 had a significantly higher ($P < 0.05$) phosphorus content and degree of substitution compared to the other distarch phosphates. Cross-linking treatment is done by producing side bonds in different chains using cross-linking reagents such as phosphorus oxychloride, epichlorohydrin, sodium tripolyphosphate and sodium trimetaphosphate (Haq *et al*., 2019). Kaur *et al*. (2006) observed that the cross-linking potato starches significantly altered their rheological behaviour. A higher degree of cross-linking caused lower peak storage modulus (G') due to the lower swelling power

leading to weak inter-granular interaction. These researchers reported that the starch physicochemical properties could be manipulated by varying the chemical nature of the reagent, the degree of substitution, starch type, reagent concentration, pH, reaction time and temperature to effect changes in the degree of cross-linking and manipulating the extent of swelling.

Table 3. Acetyl content and degree of substitution of acetylated starch.

Starch	Acetyl content (%)	DS
Tapioca starch acetates:		
TSA-1	0.498±0.05[c]	0.019±0.00[c]
TSA-2	0.680±0.04[b]	0.026±0.00[b]
TSA-3	1.770±0.13[a]	0.068±0.01[b]

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=5

Table 4. Phosphorus content and degree of substitution of distarch phosphates.

Starch	Phosphorus content (%)	DS
Native	0.053±0.001[c]	-
Tapioca distarch phosphates:		
TDP-1	0.056±0.001[c]	0.0058±0.000[c]
TDP-2	0.060±0.002[b]	0.0063±0.000[b]
TDP-3	0.077±0.002[a]	0.0081±0.000[a]

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=4

2) Swelling power

Starch acetates had significantly higher ($P < 0.05$) swelling power compared to the other starches (Table 5). Swelling of starch acetates increased with increase in acetylation but decreased in distarch phosphates with an increase in the level of cross-linking. This is due to acetyl groups in starch acetates changed the hydrophilicity and hydrogen bonding in the starch chain resulting in greater swelling of the granules (Singh *et al*., 2007).

On the other hand, cross-linking reinforced the granules by linking hydroxyl groups on adjacent polymers with covalent bonds, and thus retarding the rate of granule swelling and reducing the tendency to rupture (Breuninger *et al*., 2009). A higher degree of cross-linking (TDP-3) led to stronger bonding between the starch chains that restricted the swelling of the granules. Wongsagonsup *et al*. (2014) also observed a decrease in swelling power with increasing cross-linking in tapioca starches.

Table 5. Swelling power of native and modified starches.

Starch	Swelling power (g/g)
Native starch	17.76 ± 0.84^{d}
Starch acetates:	
TSA-1	21.37 ± 1.62^{c}
TSA-2	27.97 ± 1.24^{b}
TSA-3	33.89 ± 1.83^{a}
Distarch phosphates:	
TDP-1	19.76 ± 0.64^{cd}
TDP-2	18.89 ± 0.23^{cd}
TDP-3	10.40 ± 1.00^{e}

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=3

3) Molecular weight distribution

The molecular weight distribution of the starches is shown in Table 6. Native starch exhibited the highest proportions of high molecular weight amylopectin followed by the starch acetate with the lowest level of acetylation (TSA-1) and distarch phosphate with the lowest level of crosslinking (TDP-1). Tapioca starch differs from other starches due to its lower amylose content and high molecular weights of amylose and amylopectin (Breuninger *et al*., 2009). The content of amylose in tapioca starch varies between 18 and 24% (Charoenthai *et al*. 2018).

A consequence of starch modification is the molecular weight of amylopectin (molecular weight greater than 5×10^5) decreased and shifted to lower values due to the structural transformation of the starch chain caused by the introduction of acetyl groups in starch acetates and formation of phosphate crosslinks in distarch phosphates. Amylose should preferentially cross-link among themselves resulting in an increase in the amylose fraction (Jane *et al*. , 1992). Jane *et al*. (1992) observed the opposite effect in cross-linked potato and corn starch. They concluded that higher proportions of amylopectin fraction were due to amylose being cross-linked to amylopectin and eluted together with the amylopectin. Mua and Jackson (1997) determined that molecular weight influenced pasting properties of starches, and thus their application. High molecular weight fractions of amylopectin gave high peak temperature, low peak viscosity and lower shear-thinning values when pasted. Upon cooling, they formed weak gels and retrograded more. This would make them unsuitable for yoghurt making since they would produce low viscosity gels that were susceptible to shear and would not tolerate low storage temperatures.

Table 6. Molecular weight distribution (%) of native and modified tapioca starches.

Starch	$> 10^7$	10^6 - 10^7	5×10^5 - 10^6	10^5 - 5×10^5	$< 10^5$
Native	33.2 ± 2.10^{d}	31.8 ± 0.97^{c}	13.2 ± 0.60^{a}	18.5 ± 0.81^{a}	3.3 ± 0.52^{a}
Tapioca starch acetates:					
TSA-1	2.2 ± 0.18^{a}	31.4 ± 0.43^{c}	15.8 ± 0.39^{b}	32.1 ± 0.45^{c}	18.5 ± 0.39^{de}
TSA-2	15.2 ± 2.63^{c}	32.2 ± 0.41^{c}	16.3 ± 0.66^{b}	25.5 ± 1.47^{b}	10.8 ± 1.36^{b}
TSA-3	0.3 ± 0.45^{a}	32.1 ± 1.52^{c}	18.0 ± 1.19^{c}	34.2 ± 1.43^{d}	15.4 ± 3.66^{cd}
Tapioca distarch phosphates:					
TDP-1	12.7 ± 1.22^{b}	26.7 ± 0.74^{b}	16.8 ± 0.46^{bc}	31.2 ± 0.35^{c}	12.6 ± 0.68^{bc}
TDP-2	0.3 ± 0.52^{a}	27.5 ± 0.96^{b}	21.1 ± 1.54^{d}	39.9 ± 1.35^{e}	11.2 ± 3.43^{b}
TDP-3	0.5 ± 0.17^{a}	21.1 ± 1.45^{a}	16.5 ± 0.52^{bc}	41.5 ± 1.32^{e}	20.4 ± 0.98^{e}

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=3

4) RVA analysis

The pasting profiles and properties of native and modified tapioca starches are shown in Figure 3 and Table 7. For the starch acetates, pasting temperatures of TSA-2 and TSA-3 had significantly lower ($P < 0.05$) values than native starch. These lower pasting temperatures indicated their lower resistance towards swelling due to higher level acetylation. The reduction in the pasting temperature is an advantage of acetylation because it allows the use of acetylated starches in cases where a thickening agent must gelatinize at a lower temperature, or also to reduce energy costs during processing of products where these starches are used (Colussi *et al*., 2015). For distarch phosphates, on the other hand, cross-linking resulted in significantly higher ($P < 0.05$) pasting temperature due to cross-linking depressing the disintegration of starch granules in the swelling process which in turn increased their higher resistance swelling (Kurakake *et al*., 2009).

The peak viscosities of the starches varied significantly ($P < 0.05$) with TDP-2 having the highest value (716.39 RVU). The acetyl groups in starch acetates changed the hydrogen bonding in the starch chain resulting in greater swelling of the granules leading increased peak viscosity. In addition, the peak viscosities increased with increasing levels of acetylation. The higher peak viscosities in distarch phosphates, except for TDP-3, compared with that of native starch were due to phosphate intermolecular linkage in starch molecules restricted the swelling of the granules. TDP-3 had significantly lower ($P < 0.05$) peak viscosity unlike the other distarch phosphates and did not have a distinct peak viscosity but a continuous increase in viscosity during heating. This pasting behaviour is typical to starches with a high level of crosslinking (Thomas & Atwell, 1999).

The modified starches had significantly lower ($P < 0.05$) breakdown viscosities compared to the native. Unmodified starch granules are only held together by hydrogen bonds and are weakened by high temperatures, shear and acid (Breuninger *et al*., 2009). TDP-3 had the lowest breakdown viscosity (1.66 RVU). The low breakdown in the viscosity showed that the granules

were quite strong and resisted the breakdown under shear and heat. The starch acetates had lower setback viscosities compared to the native starch while the distarch phosphates had higher values. The acetyl groups in starch acetates restrict the tendency of the starch molecules to realign after cooling resulting in lower setback values.

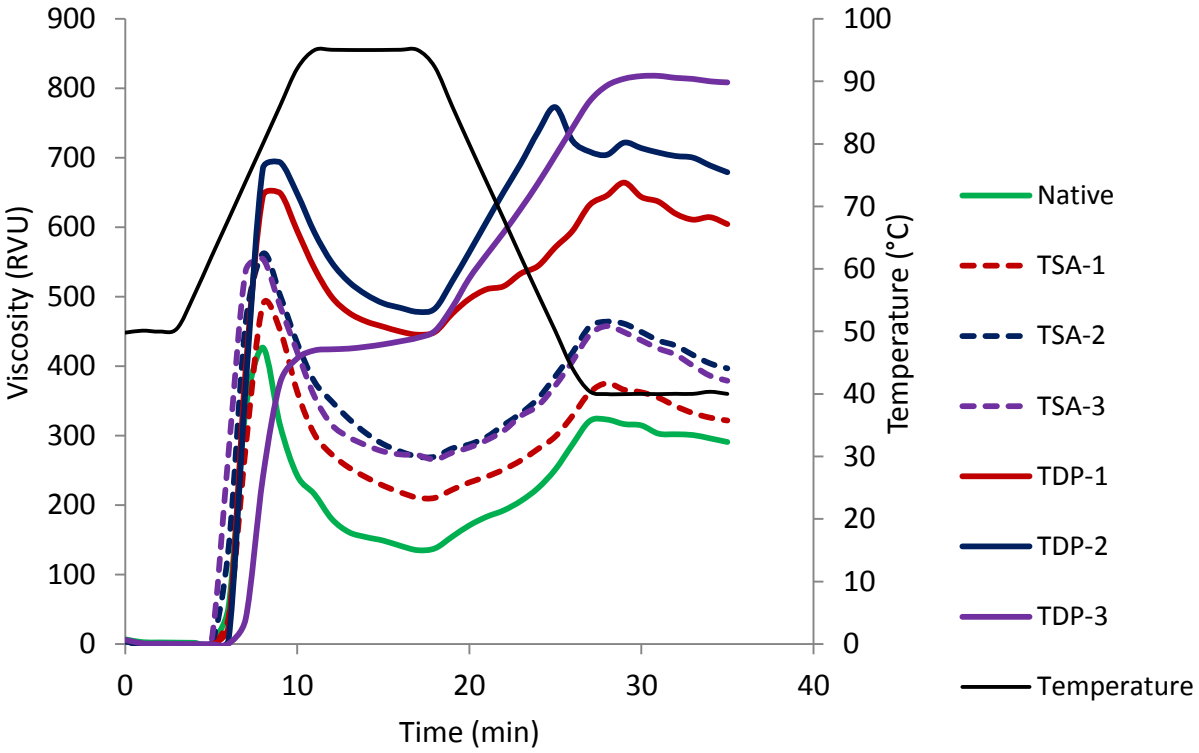


Figure 3. Pasting profiles of native and modified tapioca starches.

Starch suspensions (3 g starch in 25 g water) heated from 50°C to 95°C, held for 5 min, cooled to 50°C and held for 7 min at heating/cooling rate of 6°C/min and under constant agitation (160 rpm).

Table 7. Pasting properties of native and modified tapioca starches.

Starch	Pasting temperature (°C)	Peak viscosity (RVU)	Trough viscosity (RVU)	Breakdown (RVU)	Setback (RVU)
Native	64.8±0.20^{c}	461.67±5.63^{b}	130.1±6.15^{a}	331.58±6.21^{f}	159.28±2.23^{b}
Tapioca starch acetates:					
TSA-1	65.6±0.10^{d}	513.47±1.97^{c}	205.1±4.38^{b}	308.38±2.94^{d}	118.92±4.38^{a}
TSA-2	63.6±0.09^{b}	573.06±9.84^{d}	263.7±4.81^{c}	309.33±10.10^{d}	137.14±8.66^{a}
TSA-3	61.4±0.06^{a}	579.00±0.80^{d}	258.3±5.55^{c}	320.72±4.91^{e}	116.56±4.01^{a}
Tapioca distarch phosphates:					
TDP-1	66.4±0.08^{e}	665.66±14.81^{e}	431.8±11.24^{e}	233.83±3.75^{b}	178.55±8.98^{b}
TDP-2	67.3±0.13^{f}	716.39±7.84^{f}	471.3±3.19^{f}	245.14±4.76^{c}	210.92±6.10^{c}
TDP-3	71.2±0.19^{g}	411.08±17.64^{a}	409.4±17.72^{d}	1.66±0.09^{a}	394.67±26.04^{d}

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$

n=3

RVU= Rapid Visco Units

2.3.2. Physico-chemical properties of yoghurts

1) Chemical properties of yoghurts

Titratable acidity (TA) of the yoghurt ranged between 0.96 and 0.98% and there were no significant differences among samples (Table 8). The acidity arises as a consequence of lactic acidification obtained at the end of incubation as a result of lactose fermentation by the associative growth *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The fat content of the non-fat yoghurt ranged between 0.42 and 0.49% and there were no significant differences among samples (Table 8). Full-fat yoghurt had 3.40 % while low-fat was 1.53%.

2) Syneresis

Syneresis is a common defect during storage of yoghurt and primarily occurs due to rearrangements of aggregates of casein micelles in the gel network. Spontaneous syneresis has been attributed to slow shrinkage of the protein gel network that results in the loss of the ability to entrap all the serum phase without the application of any external forces e.g., centrifugation (Lucey, 2002). Modified starches have been used to reduce yoghurt syneresis by holding substantial quantities of water into weak gel structures (Luo & Gao, 2011). The effect of modified starch addition on whey loss of the non-fat yoghurt was therefore measured and the results are shown in Table 8. The results showed that the addition of TSA-3 starch significantly decreased the level of whey loss by 5.0% when compared to the control. This could be attributed to its relatively high content of acetyl which caused a more influential effect on starch granule swelling than cross-linking.

Table 8. Physicochemical properties of yoghurt samples.

Yoghurt samples	Titratable acidity(%)	Fat content (%)	Protein content (%)	Syneresis (%)
Full-fat	0.98±0.00[a]	3.40±0.07[a]	3.53±0.01[c]	7.51±0.83[e]
Low-fat	0.98±0.01[a]	1.53±0.14[b]	3.80±0.67[b]	14.10±1.99[d]
Non-fat	0.98±0.02[a]	0.45±0.03[c]	4.51±0.07[a]	22.81±1.27[ab]
Native starch	0.97±0.01[a]	0.49±0.00[c]	4.56±0.34[a]	23.97±0.69[ab]
Tapioca starch acetates:				
TSA-1	0.96±0.01[a]	0.42±0.04[c]	4.57±0.33[a]	23.24±0.68[ab]
TSA-2	0.98±0.00[a]	0.49±0.00[c]	4.54±0.08[a]	21.03±0.35[bc]
TSA-3	0.98±0.01[a]	0.48±0.04[c]	4.51±0.26[a]	17.83±1.28[cd]
Tapioca distarch phosphates:				
TDP-1	0.96±0.02[a]	0.45±0.08[c]	4.58±0.33[a]	24.85±1.69[ab]
TDP-2	0.97±0.02[a]	0.49±0.07[c]	4.57±0.24[a]	22.80±1.42[ab]
TDP-3	0.97±0.01[a]	0.44±0.08[c]	4.53±0.46[a]	25.48±0.88[a]

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=3

It has been reported that the introduction of acetyl groups could disrupt the hydrogen bonds in the starch granules and disorganize the intragranular structure; thereby increasing the water binding of starch chains (Sodhi & Singh, 2005). Mirmoghtadaie *et al*. (2009) and Sodhi & Singh (2005) observed that high degree of cross-linking could lead to strong bonding between the starch chains that restricts the mobility and swelling of the granules increasing syneresis. TDP-3 had the highest syneresis although not significantly different from yoghurts with native, TDP-1, TDP-2, TSA-1 starches and the control. Syneresis for the other yoghurts did not differ significantly from the control.

3) Particle size distribution

Figure 4 shows the particle size distribution of the yoghurt samples. For full-fat and low-fat yoghurts, the particle diameter ranged from 5.1 to 133.7 μm with one main peak at 30.07 μm whereas non-fat yoghurt ranged from 5.1 to 146.8 μm with its peak at 42 μm. On the other hand, particle sizes of non-fat yoghurts made with starch ranged between 4.2 and 373.1 μm and had two or three peaks characteristic. The first peak at about 15 μm, the second one at about 30-40 μm and the third one at 100 μm. Peaks at 10-50 μm come from casein-whey protein aggregates. The addition of starch resulted in the peaks shifting to larger diameters (greater than 90 μm) due to milk proteins adsorbed onto the gelatinized starch granules.

The peculiar two or three peaks characteristic behaviour of these yoghurts could be attributed to some swollen starch granules being incorporated into the gel network and while some of starch gel fragments being unevenly distributed in the yoghurt independent of the protein network (Sandoval-Castilla *et al*., 2004). The effect of starch modification and level of modification on the non-yoghurt particle sizes could, however, hardly be observed for this experiment.

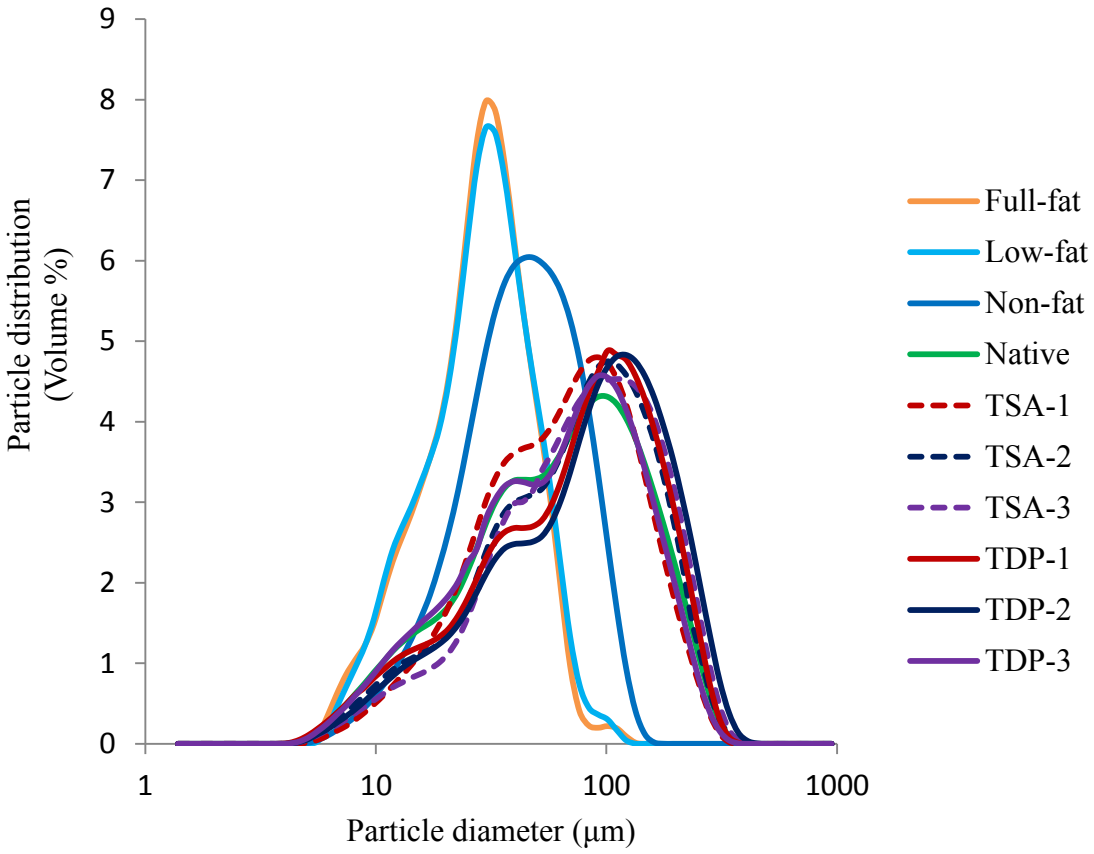


Figure 4. Particle size distribution of yoghurt samples.

Yoghurt dispersed in deionized water without ultrasonic dispersion.

4) Protein surface hydrophobicity (PSH)

The fluorescent probe binding method was used to evaluate the surface hydrophobicity of the milk proteins. The fluorescent marker ANS, a hydrophobic dye with an affinity for hydrophobic regions of proteins, was used in protein hydrophobicity studies (Bonomi *et al*., 1988). When milk protein molecules are unfolded, the exposed inner hydrophobic groups react with the ANS probe forming an ANS–milk protein" complex. Variations in fluorescence intensity and PSH values could be indirectly related to protein-starch interactions. The effect of the addition of the modified starches to non-fat yoghurts are given in Table 9 and ANS titration curves are shown in Figure 5.

Regarding $F_{max,}$ which is the maximum fluorescence that could be attained and also the maximum surface allowable for hydrophobic sites that ANS could be bound, TSA-3 yoghurt had significantly the lowest values compared to the all the other yoghurts (Figure 5). These results suggest that a decrease in F_{max} values due to the blocking of the hydrophobic surface sites by TSA starches could be associated with the existence of stronger attractive interactions between casein micelles and the starches. Chi *et al*. (2008) have noted that acetylated starches had increased hydrophobicity due to reduced hydrophilicity of esters attributed to the replacement of hydrophilic hydroxyls by the relatively hydrophobic acetyl groups. When compared to the control, a decrease in PSH indicated greater hydrophobic bonding between milk proteins and starch. The PSH decreased significantly (P<0.05) following the introduction of starch acetates (Table 9). The lowest value was obtained with TSA-2 and TSA-3. The results indicated differences exist in the macromolecular interactions, although weak, between starch chains and granules and caseins as a result of hydrophobic interactions. This showed that there might have been other interactions (such as steric stabilization, electrostatic repulsion) besides hydrophobic interactions that play a more significant role in protein-starch interactions found in these non-fat yoghurts.

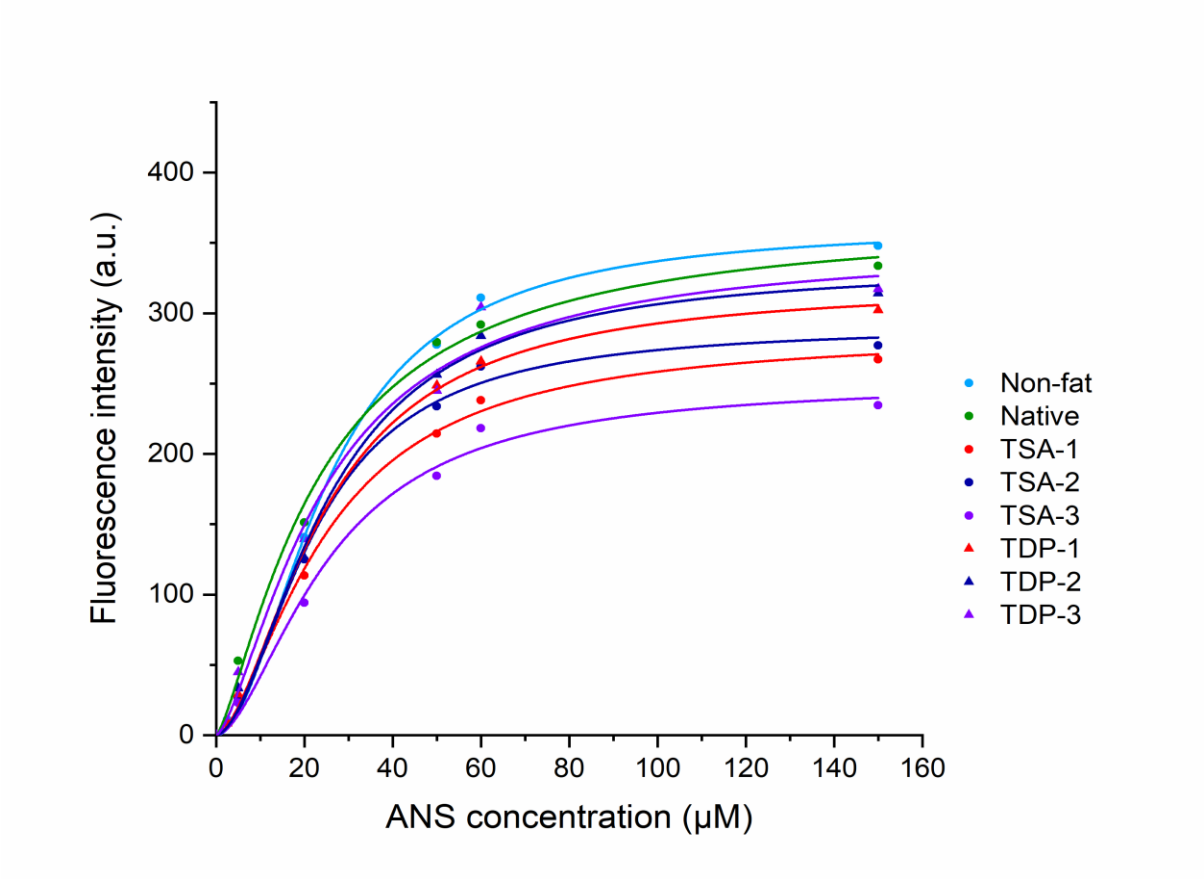


Figure 5. Changes in fluorescence intensity as a function of ANS concentration.

Yoghurts diluted with 50 mM phosphate buffer (pH 6.8) and fluorescence measured at excitation wavelength (λ_{ex}) of 390 nm and emission wavelength (λ_{em}) of 480 nm.

Table 9. F_{max}, K_d and PSH of yoghurt samples.

Yoghurt sample	F_{max}	K_d	PSH
Control	367.74 ± 21.93^{a}	23.37 ± 3.04	3.5 ± 0.02^{a}
Native tapioca	362.39 ± 17.13^{a}	25.37 ± 2.09	3.2 ± 0.00^{a}
Tapioca starch acetates:			
TSA-1	291.17 ± 15.36^{c}	22.43 ± 2.29	2.9 ± 0.00^{ab}
TSA-2	285.68 ± 18.12^{c}	24.77 ± 3.14	2.5 ± 0.00^{b}
TSA-3	251.42 ± 17.52^{c}	25.57 ± 3.41	2.2 ± 0.00^{c}
Tapioca distarch phosphates:			
TDP-1	320.33 ± 17.33^{b}	24.58 ± 0.19	2.8 ± 0.01^{ab}
TDP-2	333.75 ± 17.28^{b}	25.12 ± 2.46	2.9 ± 0.00^{ab}
TDP-3	349.12 ± 20.30^{b}	24.33 ± 2.91	3.2 ± 0.00^{a}

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.
n=3

5) Flow behaviour of yoghurt

The thixotropic behaviour of yoghurt samples as determined by the hysteresis loop is shown in Figure 7. The yoghurt samples, except those with distarch phosphates, showed a yield point followed by shear-thinning behaviour i.e., decrease in viscosity with increasing shear rate. Yoghurts with distarch phosphates exhibited shear-thickening with a slight increase in shear rate after the yield point before the onset of shear-thinning (Figure 6). During shear, break up of super aggregates occurs resulting in smaller aggregates and a decrease in viscosity with increasing shear rate is expected. Van Marle *et al*. (1999) reported that at low shear rates (< 10 s^{-1}) the super aggregates break up into small aggregates while at high shear rates (> 10 s^{-1}) breakup of the small aggregates will occur in yoghurts produced by non-ropy cultures. The considerable shear-thinning in non-fat yoghurts at higher shear rates may be attributed to the breakage of the intra and inter-molecular association (casein-casein and starch-casein) in the yoghurt. The unexpected shear thickening of yoghurts with distarch phosphates at low shear rates may be possibly due to cross-linked starches initial resistance to shear.

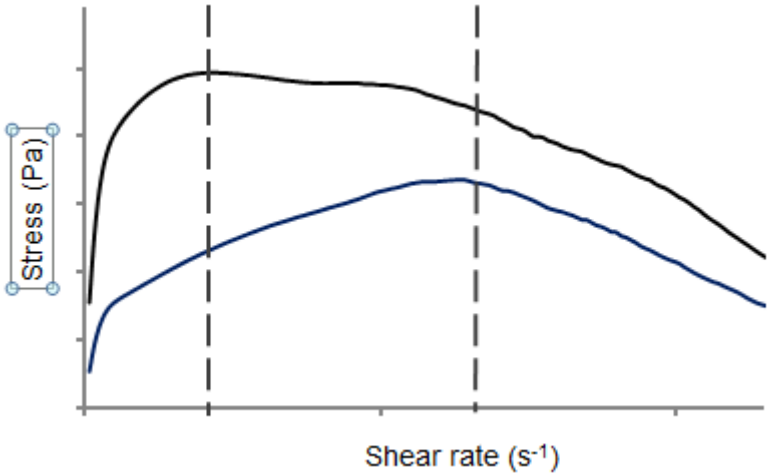


Figure 6. Representative curves showing shear-thinning after the yield point (black) and shear-thickening with a slight increase in shear rate after the yield point before the onset of shear-thinning (blue).

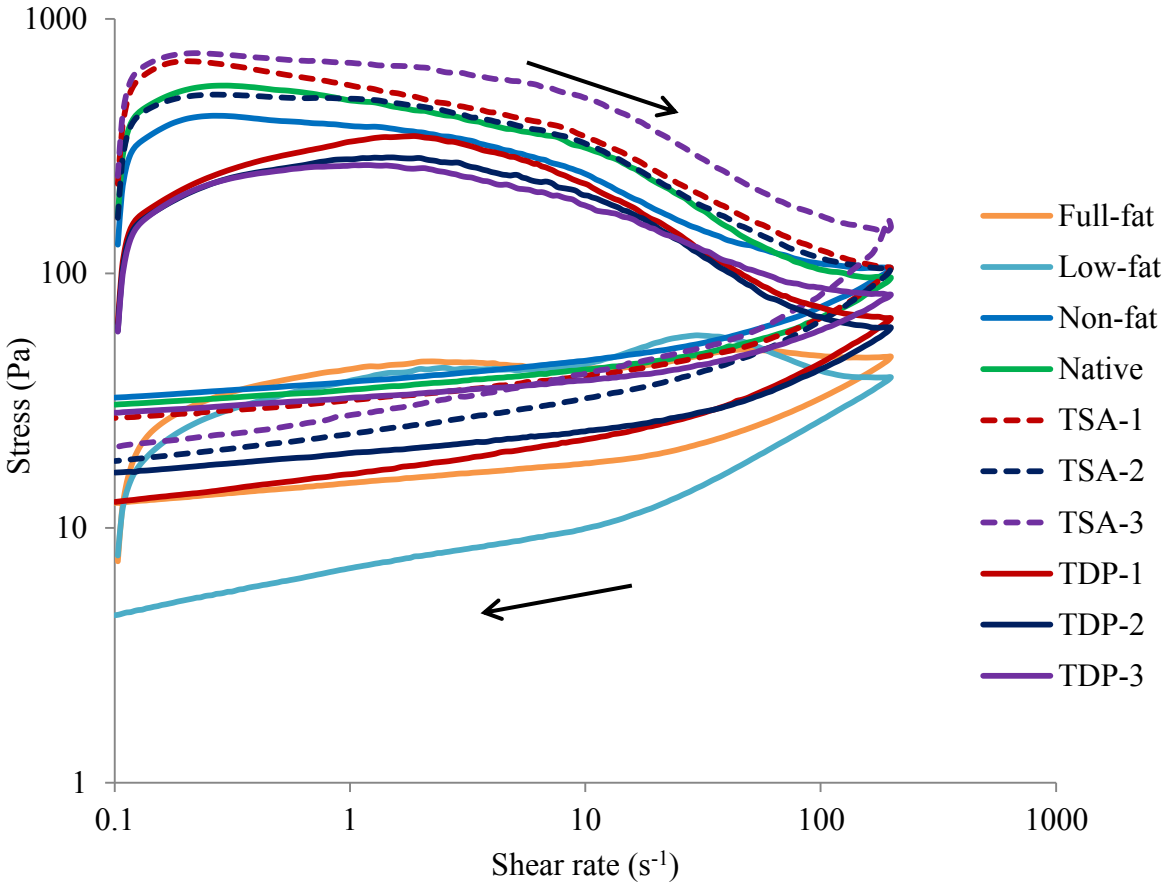


Figure 7. Flow behaviour of yoghurt samples.

Flow curves were obtained by increasing shear rates from 0.1 to 200 s^{-1} for 90 s for the upcurve and then decreasing shear rates from 200 to 0.1 s^{-1} for 90 s for the down curve at 20°C.

Flow behaviour of yoghurt can be described by yield stress (σ_0), consistency index (K) and flow behaviour index (n). Values of Herschel–Bulkley flow model parameters used to describe the up curves are summarized in Table 10. Yield stress is the minimum shear stress which is required to initiate the flow of yoghurt which characterizes the firmness of yoghurt. The consistency index is a measure of yoghurt's resistance to flow. The flow behaviour index characterizes the rheological nature of a material. Values of determination coefficients (R^2) show that the Herschel–Bulkley flow model was a good fit for the flow curves. Full-fat and low-fat yoghurts had low values for the flow behaviour properties. This can be attributed to their lower protein content (Table 8). Yoghurt structure is formed by denatured whey proteins associated with the casein micelles, therefore, the higher protein content of non-fat yoghurts would result in higher gel strength. The σ_0 and K values were observed to be higher in yoghurts with native and starch acetates. The cross-linking depressed the swelling of the starch granules and thus the viscosity was decreased, while the native and acetylated starch granules swelled to a larger size, resulting in higher viscosity (Kurakake *et al*., 2009). Values of n proved non-Newtonian flow and shear-thinning behaviour of the samples since the values were less than 1. Yoghurt made with starch acetates had the highest hysteresis loop area compared to the other samples. The hysteresis loop indicates that a structural breakdown has occurred and the hysteresis loop area may be used as an index of the mechanical stability (Benezech & Maingonnat, 1994). For yoghurts with starch acetates, the larger hysteresis loop area and the resulting high thixotropic index reflect lower mechanical stability of yoghurt.

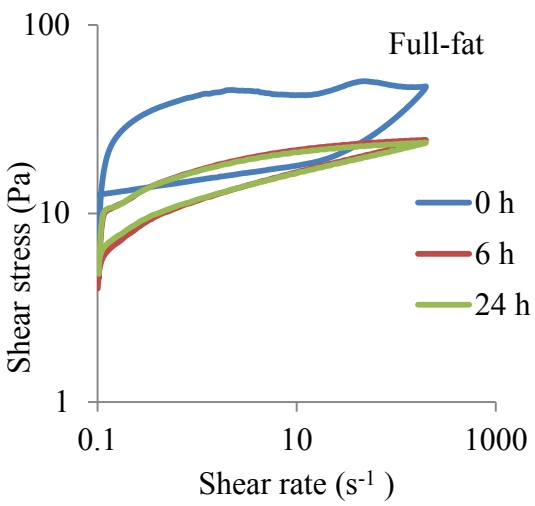
Table 10. Flow behaviour indices (n), consistency indices (K), regression coefficient (R^2), yield stress (σ_0), hysteresis loop area (A_{hys}) and thixotropic index of yoghurt samples.

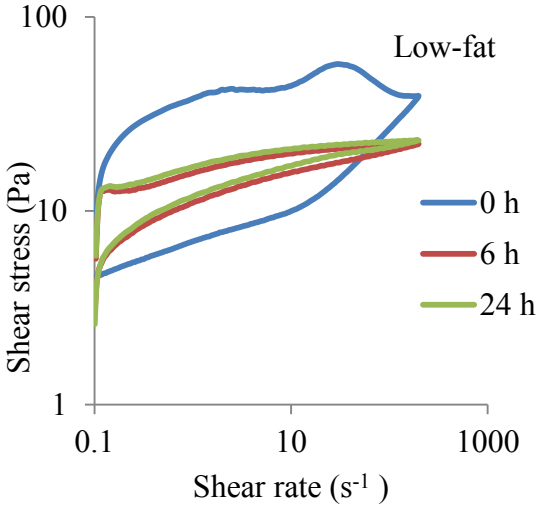
Yoghurt sample	σ_0 (Pa)	K (Pa·s^n)	n	R^2	A_{hys} (Pa/s)	Thixotropic index
Full-fat	11.53	40.74	0.26	0.97	4,962.7	0.35
Low-fat	11.35	31.62	0.20	0.97	5,938.9	0.45
Non-fat	416.28	446.68	0.30	0.98	29,676.5	0.46
Native starch	546.90	602.56	0.36	0.96	30,878.0	0.53
Tapioca starch acetates:						
TSA-1	682.00	660.69	0.35	0.97	37,893.6	0.58
TSA-2	503.91	616.60	0.34	0.97	37,380.7	0.57
TSA-3	733.38	891.25	0.33	0.95	56,945.4	0.62
Tapioca distarch phosphates:						
TDP-1	346.51	467.74	0.38	0.96	24,374.8	0.56
TDP-2	285.58	389.05	0.35	0.94	20,949.5	0.55
TDP-3	266.45	316.23	0.26	0.98	21,674.4	0.45

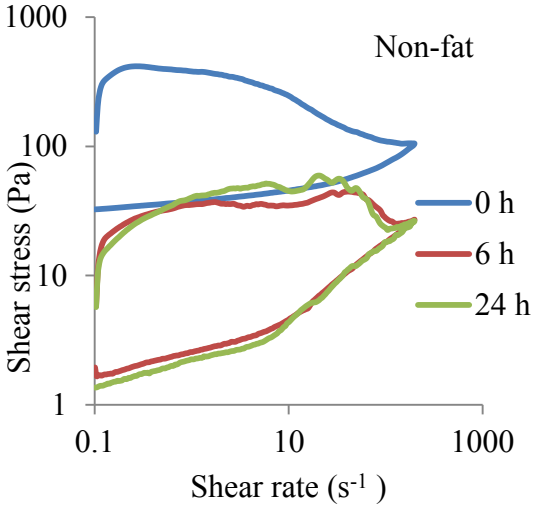
The thixotropic behaviour of yoghurt samples after storage for 6 h and 24 h was also evaluated. The yoghurt samples become thinner after stirring and did not regain their original viscosity after storage for 6 h and 24 h (Figure 8). Moreover, the hysteresis loop area did not vary significantly between the yoghurts stored for 6 h and yoghurts stored for 24 h (Table 11). Rebodying is thought to be mostly due to the production of lactic acid by bacteria after stirring, the decrease in temperature on stirring and cold storage and the production of exopolysaccharides (EPS) by bacteria during storage (Renan *et al*. 2009). In this experiment, the yoghurts were pasteurized after fermentation and non-ropy producing yoghurt culture was used indicating that cold storage alone was no sufficient for structure recovery. It appears that the interactions responsible for rebodying during storage were weak and easily broken by stirring.

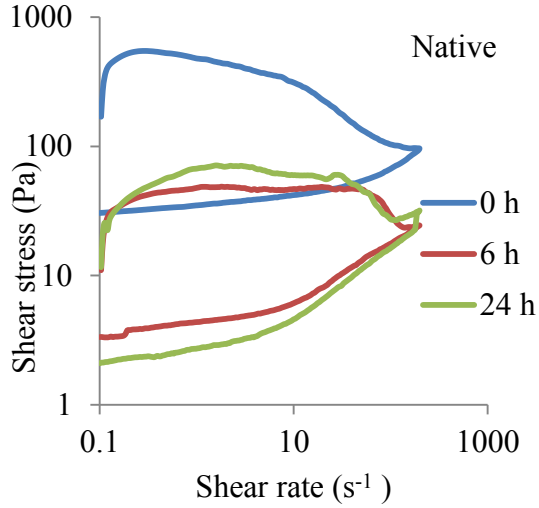
Table 11. Hysteresis loop area (Pa/s) of yoghurt samples after 0, 6 and 24 h.

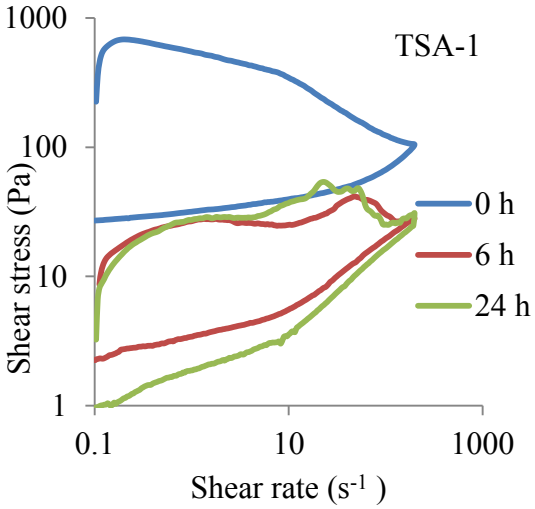
Yoghurt sample	0 h	6 h	24 h
Full-fat	4,962.7	849.4	782.5
Low-fat	5,938.9	704.0	650.3
Non-fat	29,676.5	4,722.0	5,079.8
Native starch	30,878.0	5,258.9	6,687.5
Tapioca starch acetates:			
TSA-1	37,893.6	4,039.9	5,387.8
TSA-2	37,380.7	5,049.4	5,152.2
TSA-3	56,945.4	5,301.9	6,065.2
Tapioca distarch phosphates			
TDP-1	24,374.8	5,245.3	5,462.1
TDP-2	20,949.5	5,298.5	5,099.2
TDP-3	21,674.4	4,635.7	4,746.6

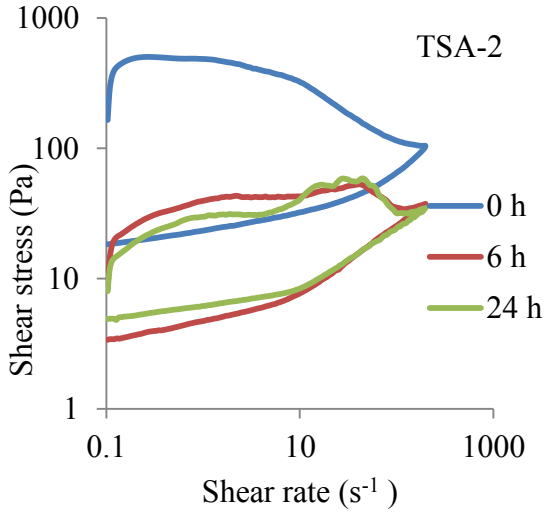

Full-fat
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h


Low-fat
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h


Non-fat
1000
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h


Native
1000
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h


TSA-1
1000
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h


TSA-2
1000
100
10
1
Shear stress (Pa)
0.1
10
1000
Shear rate (s-1)
0 h
6 h
24 h

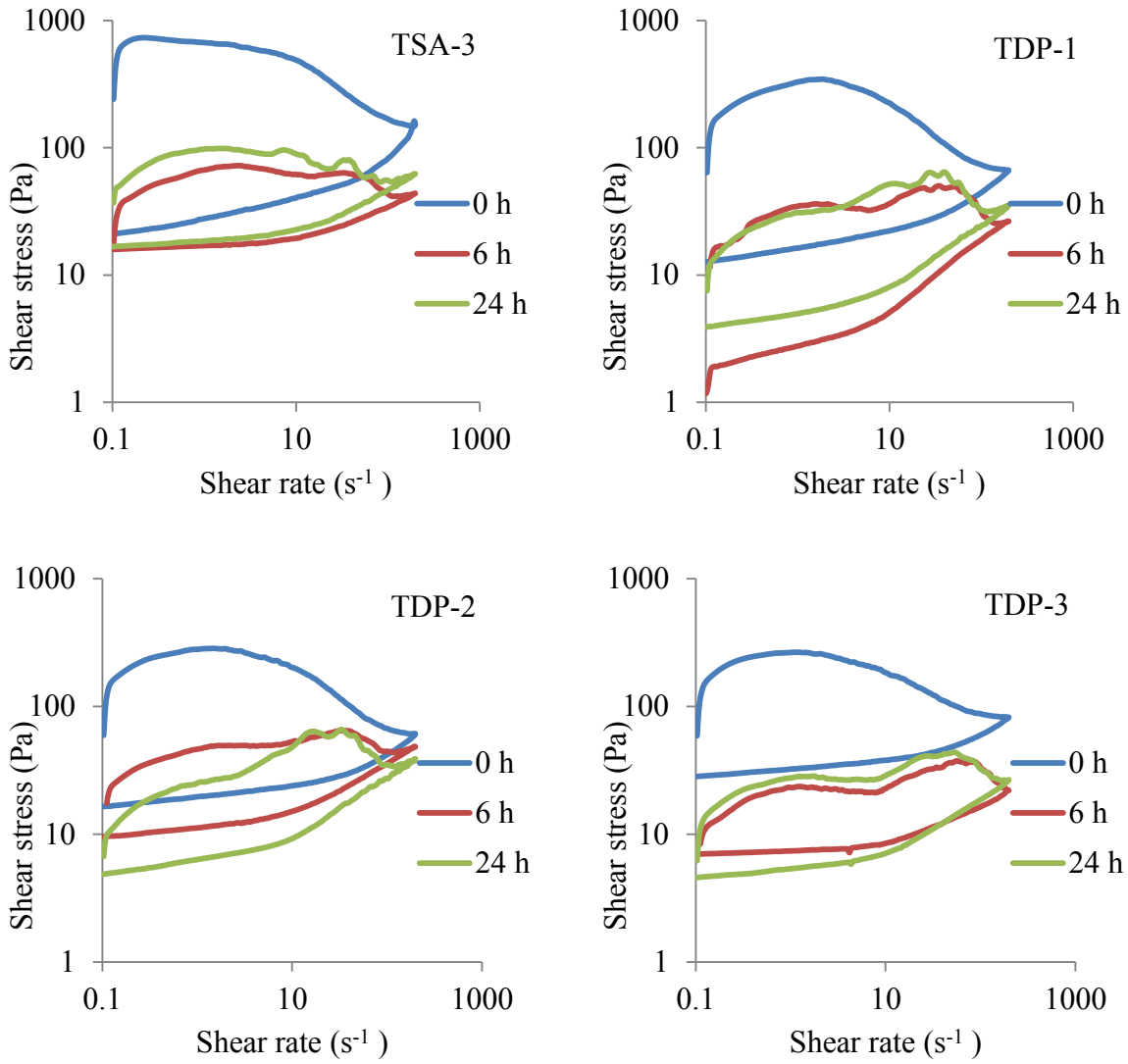
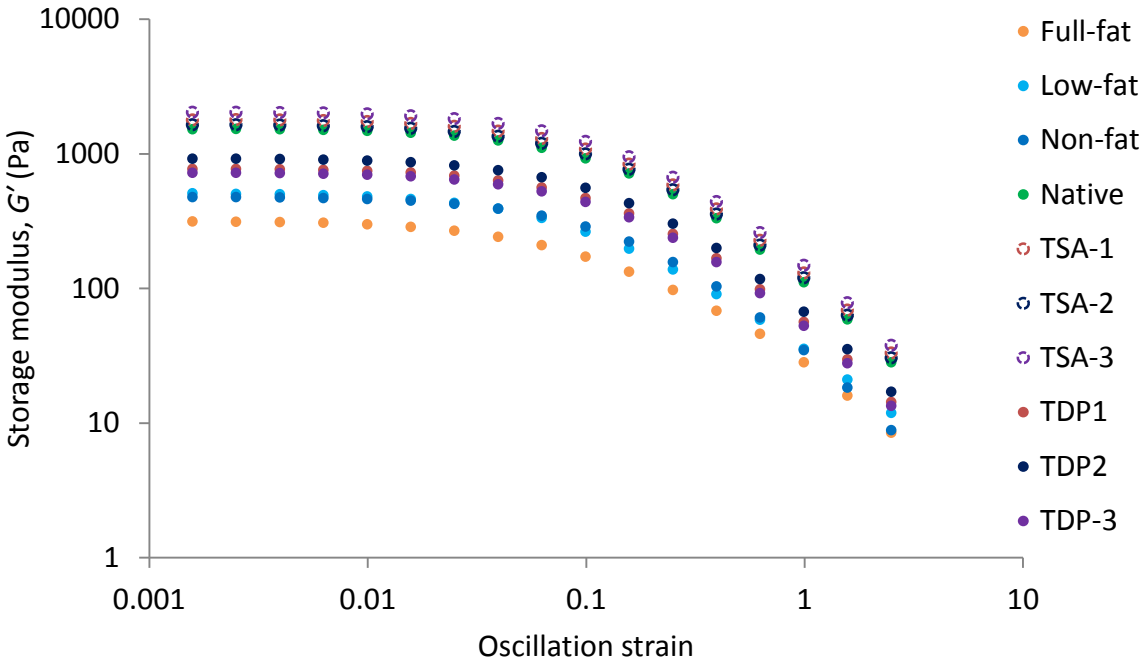


Figure 8. Hysteresis loops of yoghurt samples after 0 h, 6 h and 24 h storage time at 5°C. Flow curves were obtained by increasing shear rates from 0.1 to 200 s^{-1} for 90 s for the upcurve and then decreasing shear rates from 200 to 0.1 s^{-1} for 90 s for the down curve at 20°C.

6) Dynamic viscoelasticity testing

This test is used to determine the viscoelastic properties of food. The storage (elastic, G') modulus expresses the magnitude of the energy that is stored in the material per cycle of deformation and indicates the solid-like properties. The loss modulus (viscous, G'') is a measure of the energy which is lost as viscous dissipation per cycle of deformation and indicates the liquid-like properties (Lee & Lucey, 2010; Sharoba *et al*., 2005). The mechanical loss tangent, tan δ, which equals G''/G' and indicates the type of viscoelastic properties in a material.

Variation of the G' and G'' of the stirred yoghurt samples with oscillation strain between 0.001–2.5 was investigated to determine the linear viscoelastic range (LVE) as shown in Figure 9. From the curves obtained, the LVE limits were exceeded at the point at which the curves began to leave the constant plateau and indicate the maximum deformation tolerated by the sample before the internal structure was destroyed. The values of the critical shear strain were calculated and determined to be 0.005.



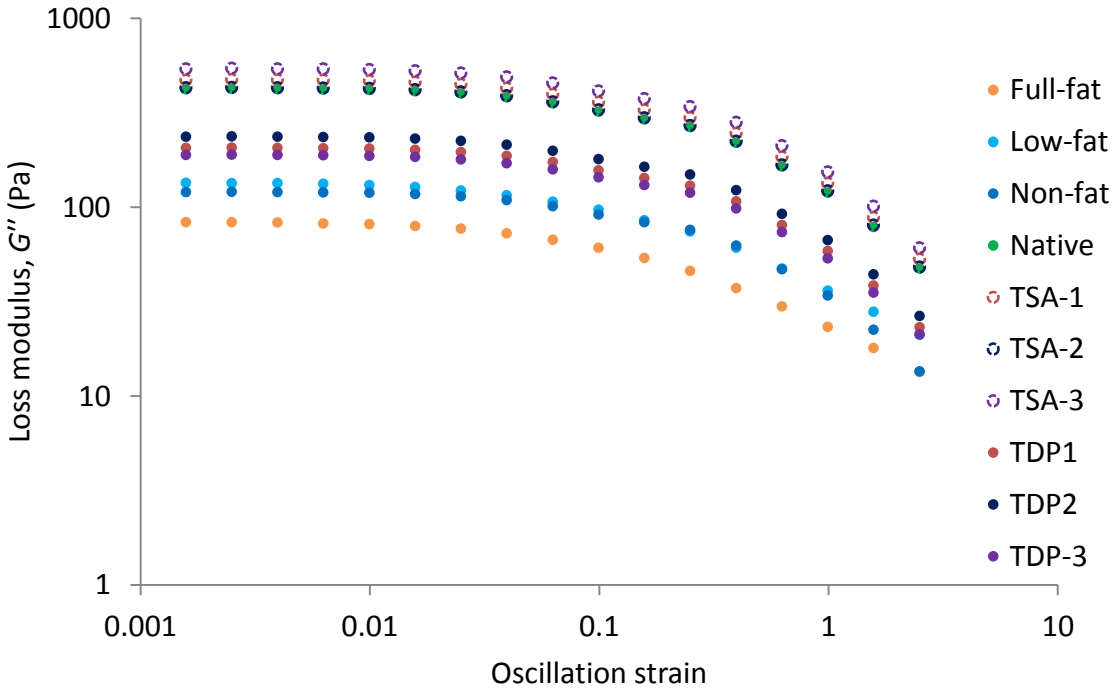
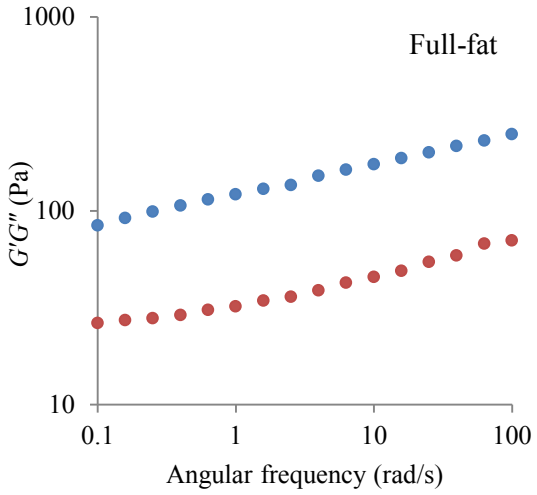


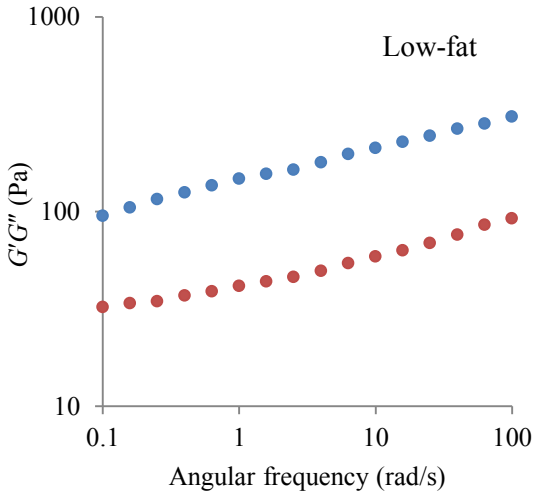
Figure 9. The viscoelastic linearity behaviour of yoghurt samples determined by amplitude sweep test.

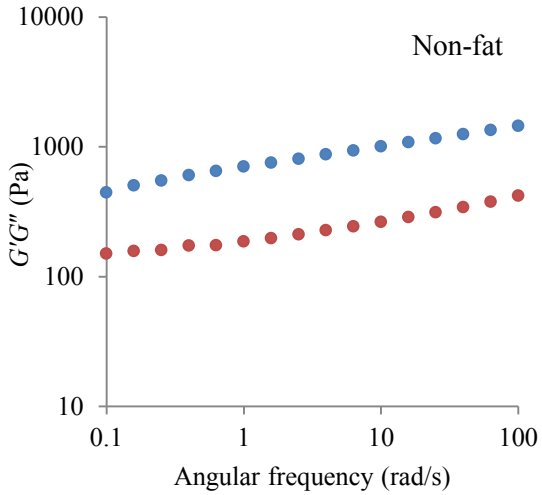
Storage modulus, G' and loss modulus, G'' of stirred yoghurts as a function of oscillation strain. Amplitude sweeps were done under an oscillation strain range of 0.001 to 2.5 at 20°C.

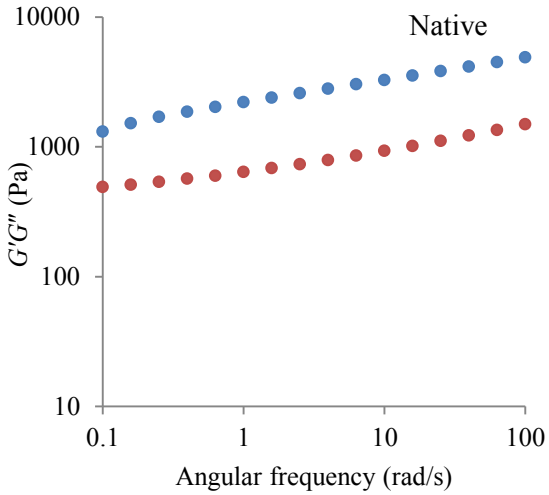
From LVE range limits, storage modulus (G') and loss modulus (G'') versus angular frequency of the yoghurt samples were evaluated at 0.005 shear strain (Figure 10). G' values were higher than G'' for all samples, indicating a typical weak viscoelastic system. G' and G'' of all the yoghurts increased with increasing frequency. Yu *et al*. (2016) observed a similar trend when they investigated the effect of milk solids nonfat (MSNF) on the physical properties and microstructure of yoghurts. Full-fat, low-fat yoghurts and non-fat yoghurt without starch had the lowest G' and G'' values whereas addition of native and modified starches resulted in higher values. During swelling, increased uptake of water by starch would result in an increase in the protein concentration in the continuous phase, leading to a stronger gel network (Bravo-Núñez *et al*., 2019). Yoghurts with starch acetates had the highest values among the non-fat yoghurts. At a higher level of starch acetylation (TSA-3), the interaction between the starch chains and particle and casein micelles formed a stronger network making the viscoelastic characteristics more significant.

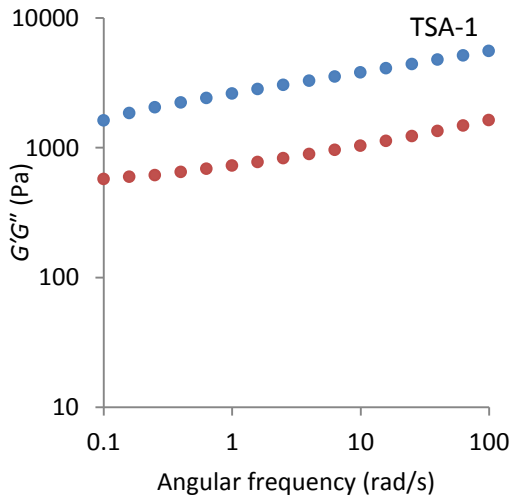
The results for mechanical loss tangent (tan δ) as a function of angular frequency are shown in Figure 11. Tan δ, calculated from G''/G', is used to interpret the viscoelastic behaviour of a semisolid food by giving a clear indication of whether the material behaviour is solid-like or liquid-like. The tan δ values higher than 1 indicated viscous behaviour and lower than 1 indicated elastic behaviour (Loveday *et al.*, 2013; Seth *et al*., 2018). In the present study, the tan δ value of all samples was less than 1, confirming all samples were more elastic than viscous, in agreement with the values of G' and G''. The results showed that the tan δ of yoghurts increased with the addition of native starch but the other yoghurts did not differ significantly.

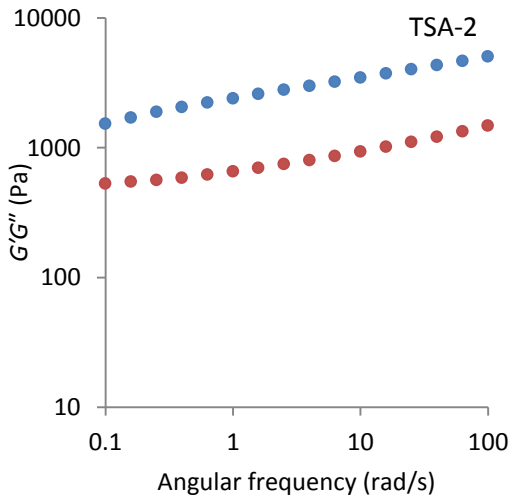

Full-fat
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)


Low-fat
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)


Non-fat
10000
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)


Native
10000
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)


TSA-1
10000
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)


TSA-2
10000
1000
100
10
G'G'' (Pa)
0.1
1
10
100
Angular frequency (rad/s)

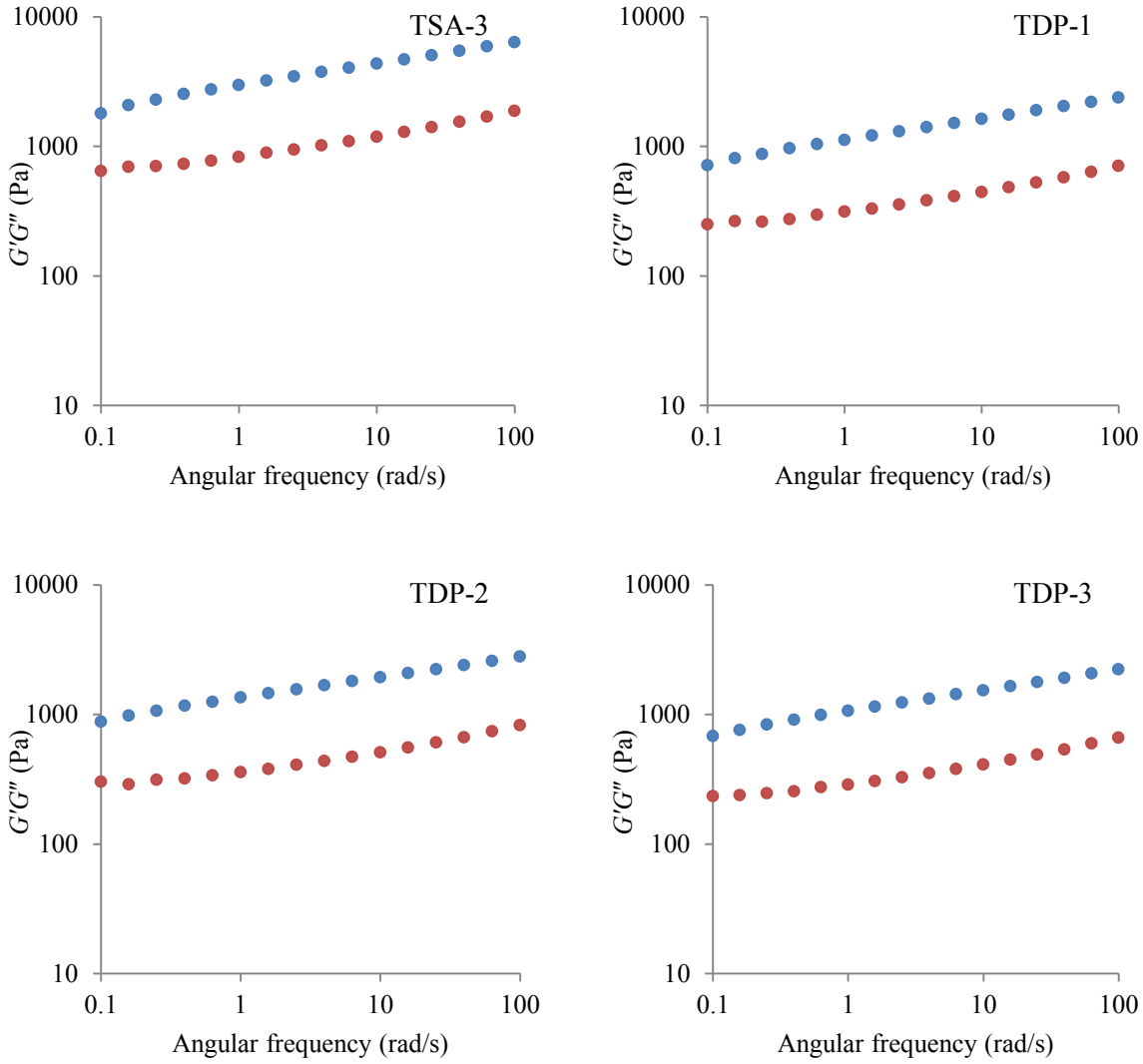


Figure 10. Storage modulus, G' (blue) and loss modulus, G'' (red) of stirred yoghurts. Frequency sweeps done under an angular frequency (ω) range of 0.1 rad/s to 100 rad/s (0.005 strain) at 20°C.

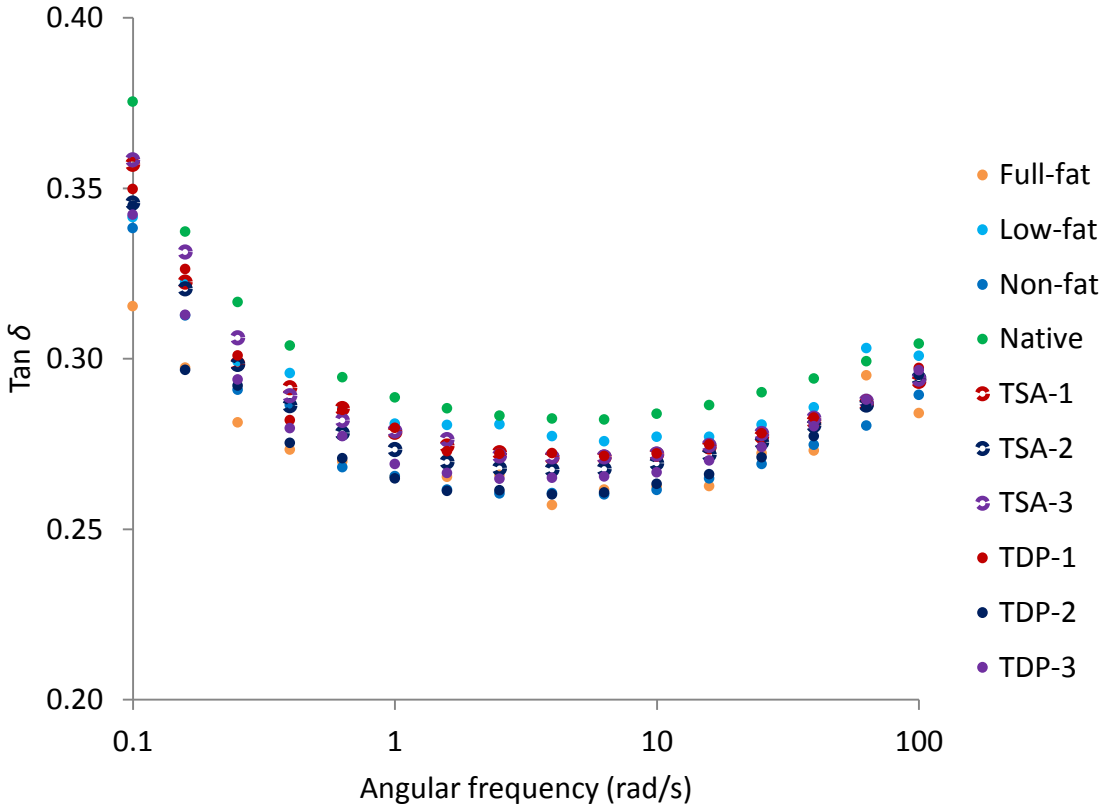


Figure 11. Tan δ of yoghurt samples.

2.4. Summary

Starch modification showed significant impact on physico-chemical properties of the starches. The degree of substitution of tapioca starch acetates (TSA-1, TSA-2 and TSA-3) was found to be 0.019, 0.026 and 0.068, respectively. As for the tapioca distarch phosphates (TDP-1, TDP-2 and TDP-3), the degree of substitution was 0.0058, 0.0063 and 0.0081, respectively. Acetylation increased the swelling power and peak viscosity of the tapioca starch, reducing its pasting temperature and reducing the tendency of retrogradation. Cross-linking starch increased pasting temperature and peak viscosity but reduced the swelling power and increased the tendency of retrogradation of the tapioca starch. These characteristics would have an impact on the final yoghurt product.

The type of starch and their level of modification were shown to significantly determine the physical and rheological properties of non-fat yoghurts. Particle size analysis showed the formation of protein-starch complexes, as evidenced by the larger particle sizes observed in non-fat yoghurts with starch, due to milk proteins adsorbing onto the gelatinized starch granules, which in turn led to a strong casein network. Although protein surface hydrophobicity results showed low hydrophobic interaction values, it can be assumed that other types of interactions such as electrostatic interaction and disulphide bonding had a more significant impact on protein-starch interaction. For the non-fat yoghurts, the syneresis and rheological properties were improved in yoghurts with starch acetates compared to the control and yoghurts with distarch phosphates and native starch. These yoghurts had lower syneresis and improved viscosity and moduli due to greater interaction between starch chains and casein micelles and the presence of these starches in the serum phase. By increasing the acetylation level of starch acetates, a decrease in syneresis and improved rheological properties were seen due to a higher number of acetyl groups incorporated that caused more disruption in the intragranular structure. This, in turn, increased the water binding of starch granules during the heat treatment. Contrary to this, the addition of distarch phosphates to non-fat yoghurt resulted in high syneresis and poor

rheological properties. By increasing the level of crosslinking, the non-fat yoghurts had even greater whey separation and lower viscosity. A higher degree of cross-linking led to stronger bonding between the starch chains that restricted the swelling of the granules and thus, higher syneresis.

Chapter 3

Effect of the interactions between milk proteins and native and modified starches on the microstructure of non-fat yoghurts

3.1. Introduction

Confocal laser scanning microscopy (CLSM) allows samples to be observed with few preparation procedures, due to its unique optical sectioning and high-resolution abilities (Lucey *et al*., 1998). It has often been used to image food emulsions and gels, where the microstructure is distorted or destroyed by other microscopy preparation techniques. CLSM has been used to investigate the microstructure of fermented milk gels and yoghurts in several studies including Lucey *et al*., 1998; Skytte *et al*., 2015 and Pang *et al*., 2019. Lee and Lucey (2010) attributed this irregularity in the microstructure of stirred yoghurts is most likely a consequence of the stirring process that destroys the more homogeneous network of intact yoghurt gels. CLSM micrographs produced can be used to visualize changes in the microstructure of yoghurts.

The results of many studies evaluating the microstructural properties of stirred yoghurts are mostly descriptive and qualitative. However, little information is available on the quantitative description in terms of the structure and the interactions between aggregates of casein micelles.

CLSM makes it possible to obtain a series of two-dimensional images (x, y) by z-stacking (Moussier *et al*., 2019). Using the appropriate software, these images can be compiled and computed into a 3D representation. These researchers found that estimations of the fractal dimension were more reliable and accurate using 3D than 2D even though 2D was faster. 3D reconstruction can also be used to obtain information on the structure of the samples using the mass fractal dimension (D_f). Calculation of the fractal dimension has been successfully applied to examine the aggregation behaviour of proteins and the final structure in gel systems (Skytte *et al*., 2015). One way of using fractals as a quantitative technique is to merge the use of the box-

counting technique. Therefore, the study of the fractal structure of these protein gels was used to establish a link between changes in formulation and final mechanical properties. However, only a few studies on fractal analysis exist on the microstructure of non-fat yoghurt in which modified starch have been incorporated as used as fat replacers. To better understand the interactions between milk proteins and modified starches, CSLM experiments were conducted.

3.2. Materials and Methods

3.2.1. Materials

Fluorescein 5-isothiocyanate (FITC) and Rhodamine B were obtained from Sigma-Aldrich Corp. (St. Louis, USA) whereas Nile red was purchased from FUJIFILM Wako Pure Chemical Corp. (Tokyo, Japan).

3.2.2. Confocal scanning laser microscopy (CSLM)

The microstructure of the yoghurts was studied using a confocal laser scanning imaging system (C2Si, Nikon Instruments, Japan) equipped with an inverted Nikon Eclipse Ti microscope. Dual labelling of yoghurt was done to visualize the fat and protein phases in full-fat and low-fat yoghurts, and starch and protein phases in non-fat yoghurts. For staining, 0.2% Nile red, 0.25% Fluorescein 5-isothiocyanate (FITC) and 0.1% Rhodamine B in ethanol were prepared. Nile red will stain fat, FITC will preferentially stain starch and Rhodamine B will preferentially stain protein. Yoghurt (1 mL) sample was stained with 10 µL Nile Red or 10 µL FITC for 10 min followed by addition of 10 µL Rhodamine B for 10 min. An aliquot (35 µL) of the sample was put on the glass slide and covered with the cover glass. The excitation/emission wavelength for FITC was 488 nm and Rhodamine B and Nile red was 561 nm. CLSM images were acquired in 512×512 pixel resolution, at 1× zoom factor using 20× objective lens.

3.2.3. Image analysis of CLSM micrographs

Confocal 2D and 3D images were obtained using NIS-Elements AR software (Nikon, Japan). The 3D images were captured as vertical stacks (z-stacks), a series of 2-D images with step size of 0.5 µm along the z-axis. These images were processed with ImageJ software (version 1.52q, National Institutes of Health, USA). Thresholding was carried out on binarized 2D images using the IsoData setting and then used to calculate the aggregate counts, the average size and fraction of aggregates with respect to the total sample area. Fractal dimension (D_f) values from the 3D

images were then obtained by using the box count method using BoneJ pluggin in ImageJ software. This is done by placing various sizes of grids on the image and the number of boxes containing the pixels of the object is calculated on each grid size. The log-log graph of the box count against the grid size is then plotted and the slope of the graph is the fractal dimension (D_f) value.

3.2.4. Statistical analysis

IBM *SPSS* Statistics 21 software (IBM Corp., USA) was used to perform statistical analyses in the same way as 2.2.6. Analysis of variance (ANOVA) and Tukey's HSD test were performed to determine significance at $P < 0.05$.

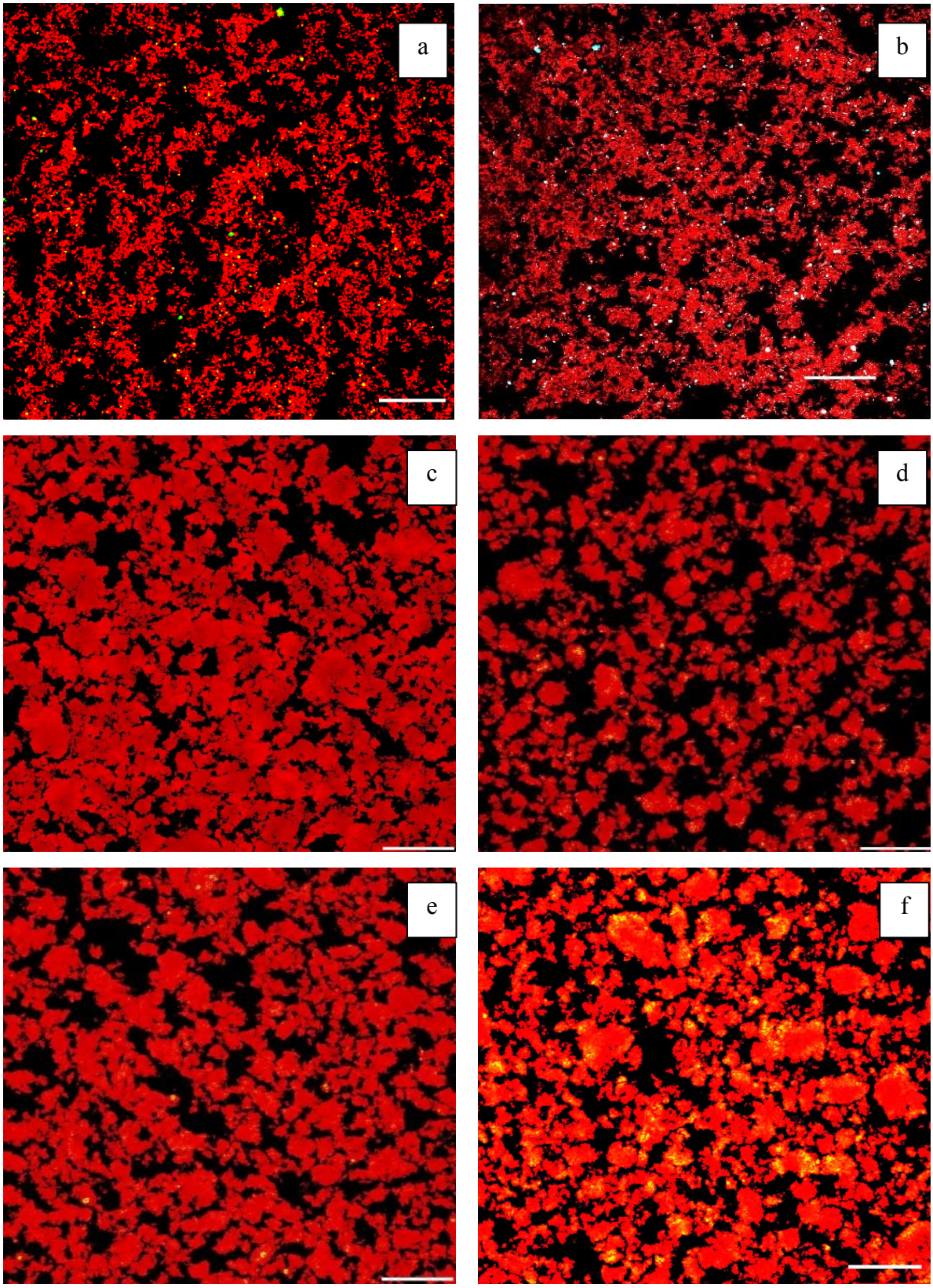
3.3. Results and Discussion

3.3.1. Yoghurt microstructure

The gross morphological differences between yoghurts are shown in Figure 12 showed that the protein aggregates (red) were embedded with starch granules (yellow) as part of the milk–starch gel network. The full-fat and low-fat yoghurts appeared to have a more homogeneous microstructure with well-distributed protein connections, whereas the non-fat yoghurt prepared with starch had densely packed protein in the form of large aggregates surrounded by an aqueous region and with fewer connections between the aggregates. The images showed that the presence of protein aggregates and low-fat content led to the formation of an interrupted and coarse gel microstructure characterised by larger void spaces (Figure 12a-c). In the case of non-fat yoghurts, the starch acetate-added yoghurts (Figure 12d-f) had a higher number of aggregates as well as less porosity in the casein network when compared to the native and distarch phosphate-added yoghurts. Yoghurts with distarch phosphates and native starch (Figure 12g-j) had large areas of separated whey and a denser protein network containing fewer aggregates.

The results on non-fat yoghurts with starch acetates (higher G″, higher viscosity, reduced syneresis) indicate that starch acetates interacted with the protein aggregates, mainly caseins, leading to a denser network. In addition, due to the acetyl groups in these starches, greater swelling of the granules enhanced water binding of starch located in the serum phase leading reduced syneresis. A different, opposite, behaviour was observed with distarch phosphates in non-fat yoghurt. The microstructure indicated significant whey separation, probably driven by limited interactions between proteins and crosslinked starches, leading to a weak network, lower G″ and viscosity and higher syneresis compared to the non-fat yoghurts. Cross-linking starch restricted swelling of the starch granules which reduced water binding of the starch leading to higher syneresis. Sandoval-Castilla *et al*. (2004) studied the microstructure of reduced-fat yoghurts made with modified tapioca starch as well as other whey protein-based fat replacers under scanning electron microscopy. They found that some solubilized starch molecules were

incorporated into the casein micelle network while starch gel fragments formed independent structures to the protein network. The microstructure results correlate well with results from syneresis and rheological tests. Reduced syneresis and increased viscosity were demonstrated by non-fat yoghurts with starch acetate.


a
b
c
d
e
f

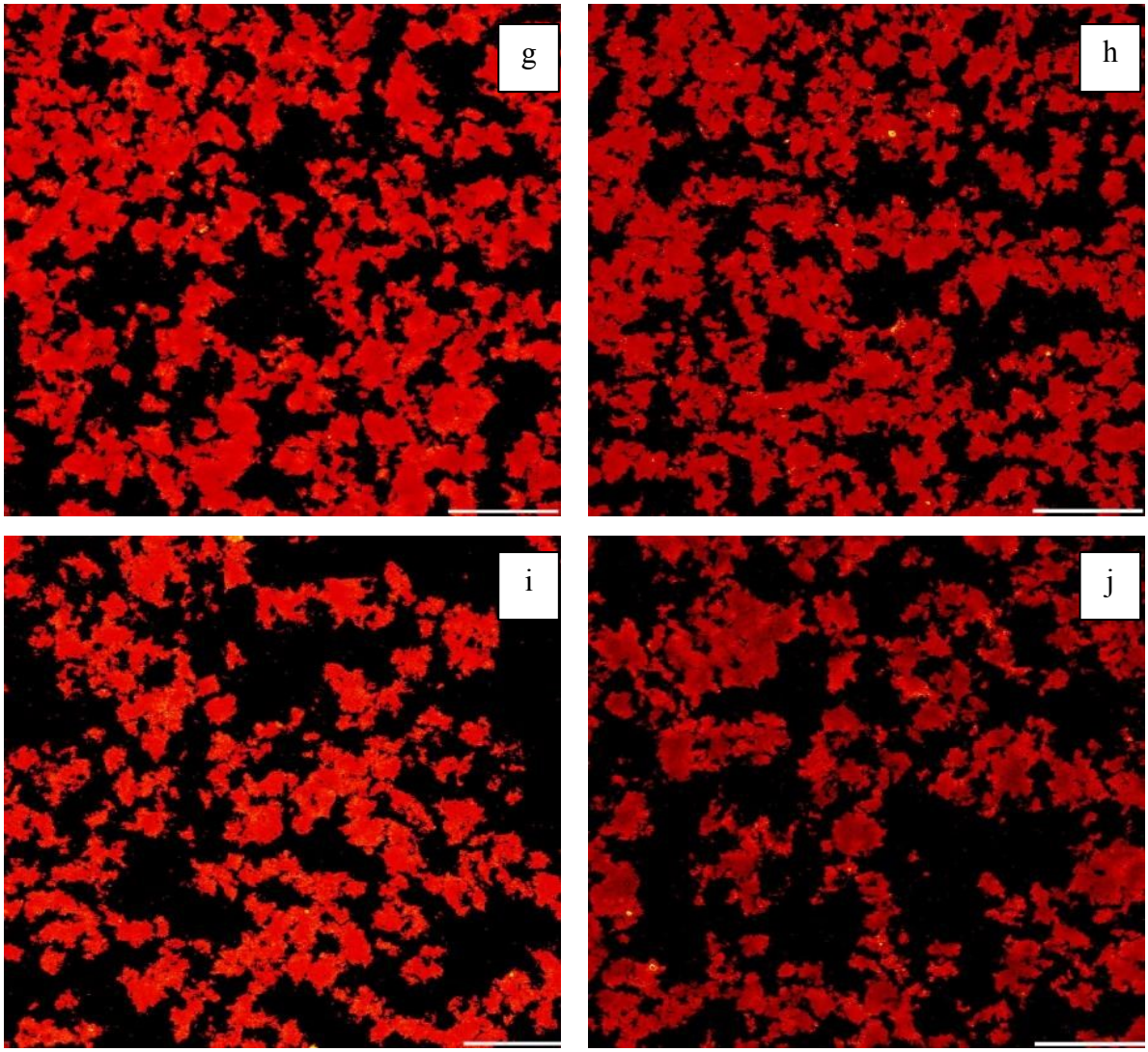


Figure 12. Confocal laser scanning micrographs showing gross morphology of stirred yoghurts prepared with; no starch added (a) full-fat milk, (b) low-fat milk, (c) non-fat milk and starch added to non-fat yoghurts (d) TSA-1, (e) TSA-2, (f) TSA-3, (g) TDP-1, (h) TDP-2, (i) TDP-3 and (j) native tapioca starch. Scale bar represents 100 μm.

3.3.2. Image analysis of CLSM micrographs

Each micrograph underwent a thresholding process where the grayscale image is transformed into a binary image with all structural features contributing white pixels and all background features contributing black pixels (Figure 13).

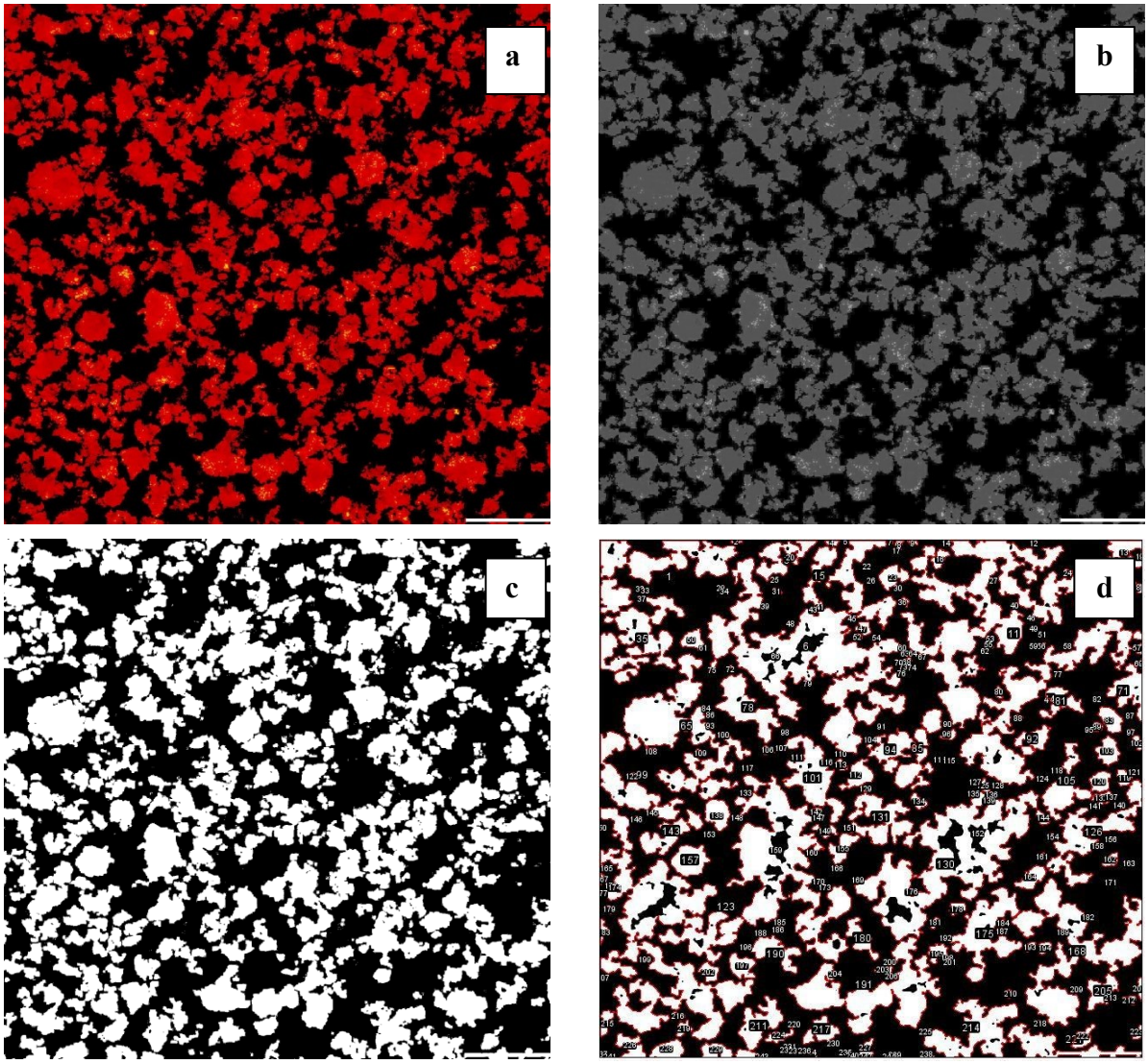
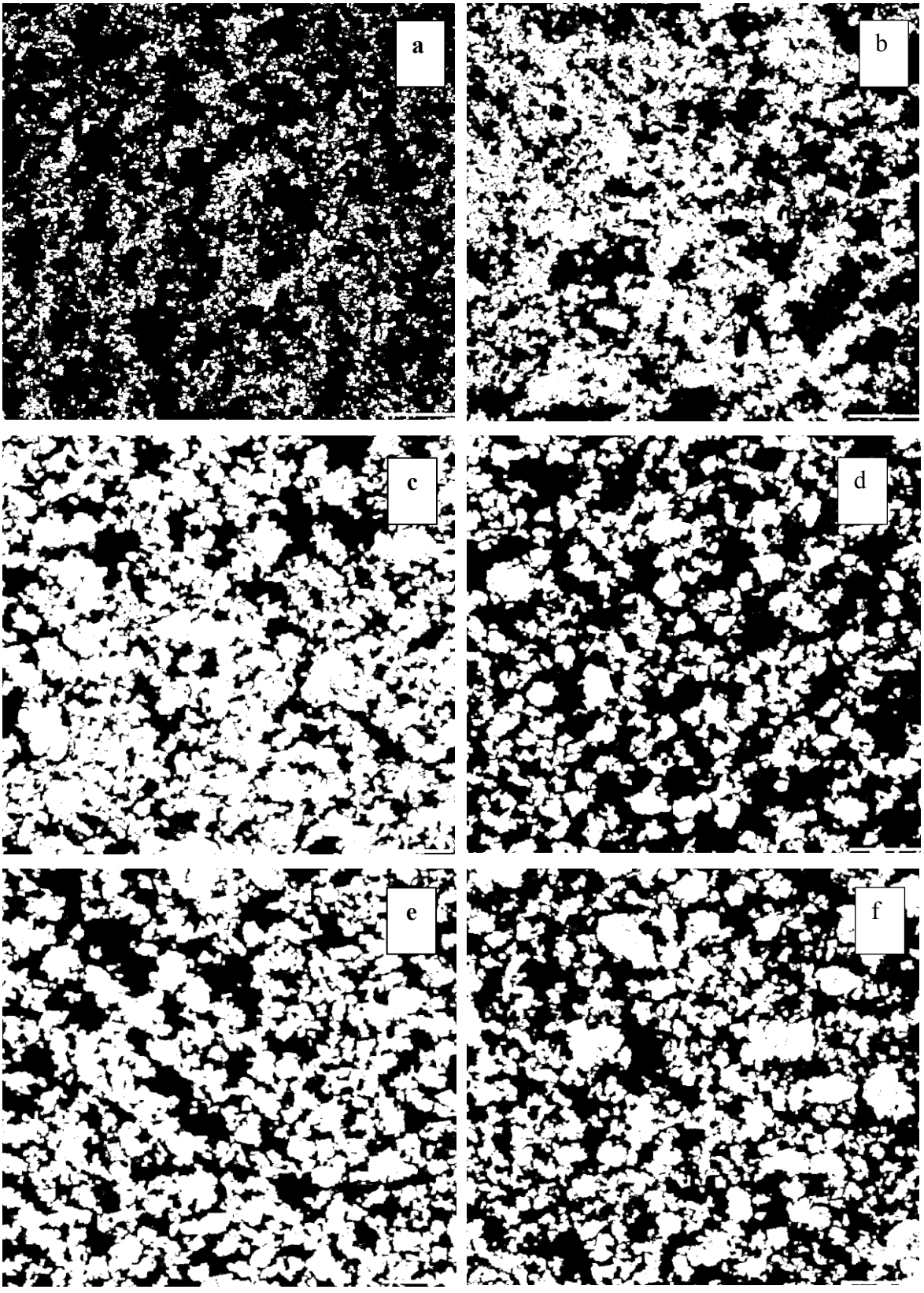


Figure 13. Representative two-dimensional images of TSA-1 yoghurt showing the image analysis process (a) original image, (b) grayscale image, (c) binary image and (d) segmented image for area fraction calculation.


a
b
c
d
e
f

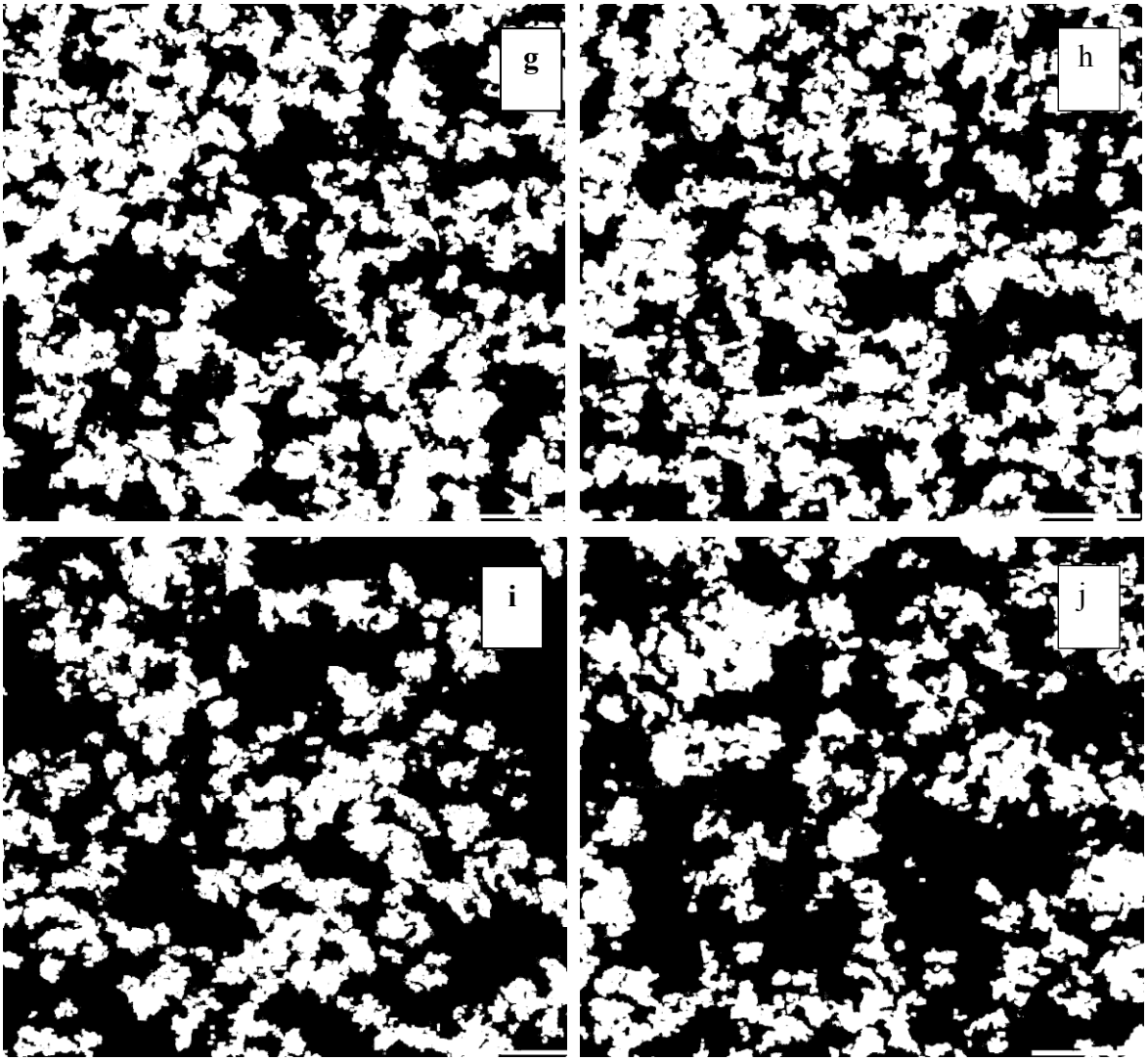


Figure 14. Binary images of CLSM micrographs showing gross morphology of stirred yoghurts prepared with; no starch added (a) full-fat milk, (b) low-fat milk, (c) non-fat milk and starch added to non-fat yoghurts (d) TSA-1, (e) TSA-2, (f) TSA-3, (g) TDP-1, (h) TDP-2, (i) TDP-3 and (j) native tapioca starch.

The higher the fat level, the more interspaced voids in the network were observed resulting in the low aggregate areas and more aggregates (Table 12). In non-fat yoghurts, starch addition increases the volume fraction of the aqueous phase, which consequently increases the mean distance between the casein micelles and so increases the extent of the aggregation of the casein micelles. In non-fat yoghurts, it is evident that the addition of starch decreased the aggregate area and increased the number aggregates, especially for TSA-2 and TSA-3. Therefore, starch acetates could have enhanced the degree of interactions in the protein network by forming links between protein aggregates resulting in a higher number of aggregates than the other non-fat yoghurts. The average aggregate sizes were significantly higher in non-fat yoghurts with starch, consistent with results obtained from particle size analysis.

Depending on the strength of the links between the aggregates, gels can be grouped into two types of behaviour: strong-link behaviour (D_f values of 2.0-2.2) and weak-link behaviour (D_f values of 2.2-2.7) (Shih *et al*., 1990). The D_f values generated for the yoghurts were in the range of 2.27–2.63 (Table 12). D_f values for all the yoghurts fall under the weak-link regime reported for protein gels by Andoyo *et al*. (2018). The results show that the highest D_f values were obtained for yoghurts with starch indicating they might act as a structure breaker because of their inability to form a cohesive network with casein.

Table 12. Protein aggregation of yoghurt samples.

Yoghurt samples	Aggregate count	Aggregate average size (μm)	Aggregate fraction (%)	D_f
Full-fat	1076±53.8[a]	34.15±7.53[d]	33.93±0.08[c]	2.27
Low-fat	865±38.0[b]	33.53±7.11[d]	73.52±0.14[a]	2.38
Control	183±45.7[d]	48.33±6.86[c]	70.41±1.63[a]	2.42
Native tapioca	258±27.7[cd]	115.43±12.86[a]	36.34±0.05[c]	2.53
Tapioca starch acetates:				
TSA-1	268±26.4[cd]	95.02±4.77[a]	53.69±0.01[b]	2.63
TSA-2	417±23.5[c]	104.10±12.03[ab]	60.09±0.29[ab]	2.56
TSA-3	555±17.2[c]	100.54±1.76[ab]	66.06±7.77[ab]	2.55
Tapioca distarch phosphates:				
TDP-1	269±19.9[cd]	109.92±3.87[ab]	52.57±2.30[b]	2.44
TDP-2	271±30.1[cd]	106.70±7.16[ab]	51.47±0.07[b]	2.48
TDP-3	258±29.6[cd]	110.82±6.92[ab]	35.82±0.11[c]	2.42

Values followed by the same superscript letter in the same column, for each measured parameter, are not significantly different at $P > 0.05$.

n=5

3.4. Summary

Fat content and the type of starch were shown to be the dominant factors for determining the microstructure of yoghurt. The micrographs of full-fat and low-fat yoghurts showed homogeneous structure with well-distributed protein connections, however, the non-fat yoghurts had densely packed protein in the form of large aggregates surrounded by an aqueous region and with fewer connections between the aggregates. The higher level of interactions occurring in the gel network also contributed to the less dense gel network with smaller pores, thereby reducing syneresis. The yoghurt microstructure was significantly affected by the type of starch and their interactions with milk proteins. Addition of native and distarch phosphates (especially TDP-3) led to the formation of an interrupted and coarse gel microstructure characterised by larger void spaces compared to yoghurts with starch acetates. This resulted in lower viscosity, high syneresis and lower moduli.

Conclusion

Overall, this research demonstrates that the type of chemical modification of starch and the level of modification affect the quality of non-fat yoghurt. Incorporating starch acetates induced positive impact on syneresis, flow and viscoelastic properties and microstructure of non-fat yoghurt while an adverse effect was observed with the addition of distarch phosphates when compared to non-fat yoghurt. In addition, the modified starches induced very different microstructure in the non-fat yoghurt. Starch acetates could have enhanced the degree of interactions in the protein network by forming links between protein aggregates resulting in a higher number of aggregates than the other non-fat yoghurts and low serum separation. This led to low syneresis and higher viscosity and moduli values.

This study illustrates that chemically modified starches can serve as functional ingredients in non-fat yoghurt production to improve texture and reduce syneresis, but care has to be taken in the choice of the type of modification as well as the level of modification. TSA-3 starch, with the highest level of acetylation, therefore, is the most suitable stabilizer in non-fat stirred yoghurt. It had lower syneresis and better rheological properties as a result of interaction between the milk proteins and starch chains. Yoghurts with distarch phosphates had poor rheological properties and higher syneresis. This indicates that these cross-linked starches are not suitable for stirred non-fat yoghurts since they do not improve characteristics and deteriorated to quality of non-fat yoghurt.

Future research work

Future research in this area is to conduct Small-Angle X-ray Scattering (SAXS) analysis in order to explore more detailed structural characteristics of casein micelles at the nanometer scale. It could also be interesting to look into the casein micellar structure to see which changes happen in the various yoghurts with different modified starches added and relate this with rheological properties and microstructure.

Ethical statements

Conflict of Interest: I declare that I do not have any conflict of interest.

Ethical Review: This study does not involve any human or animal testing.

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