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MICROSTRUCTURE AND RHEOLOGY OF YOGURT ADDED WITH PROTEIN-*L. plantarum*-POLYSACCHARIDE COACERVATE AND STEVIA IN SUBSTITUTION OF MILK-FAT AND SUCROSE

MICROESTRUCTURA Y REOLOGÍA DE YOGURT ADICIONADO CON COACERVADO DE PROTEÍNA-*L. plantarum*-POLISACÁRIDO Y STEVIA EN SUSTITUCIÓN DE GRASA LÁCTEA Y SACAROSA

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Abstract

In this work, stirred yogurt variations in which milk-fat was replaced by a complex coacervate (CC) made up by whey protein isolate/*Lactobacillus plantarum* (Lp)/ κ-carrageenan, and sucrose by stevia were prepared. Microstructure, rheology and sensory attributes of the yogurt variations were examined. Sucrose substitution (6 wt%) by stevia (0.02 wt%) in full-fat yogurt (2.6 wt%) and reduced-fat yogurt (1.3 wt%) produced more compact gel networks in which the presence of non-micellar material was observed between casein clusters. Viscoelastic moduli of the yogurt variations containing stevia were significantly higher than those of the yogurt variations containing sucrose. Incorporation of CC (1.3, 2.6 and 3.9 wt%) produced reduced-fat yogurt variations exhibiting a progressively more compact protein network, higher viscoelastic moduli and preference sensory scores comparable to those displayed by the full-fat yogurt made with sucrose. Yogurt variations incorporating CC exhibited high probiotic survivability (> 8.1 log cfu g^{-1}) after 21 days of storage.

Keywords: yogurt, complex coacervate, stevia, sucrose, *L. plantarum* survivability, rheology, microstructure.

Resumen

En este trabajo se elaboraron yogures batidos en los cuales la grasa láctea se sustituyó por un coacervado complejo (CC) de aislado de proteína de lactosuero/*Lactobacillus plantarum* (Lp)/κ-carragenina y la sacarosa por stevia. La microestructura, reología y atributos sensoriales de los yogures fueron evaluados. La sustitución de sacarosa (6 % p/p) por stevia (0.02 % p/p) en yogures completo (2.6 % p/p) y reducido (1.3 % p/p) en grasa produjo matrices geladas más compactas en donde se observó la presencia de material no micelar entre los agregados de caseína. Los módulos viscoelásticos de los yogures conteniendo stevia fueron significativamente mayores que aquellos de los yogures conteniendo sacarosa. La incorporación de CC (1.3, 2.6 y 3.9 % p/p) al yogurt reducido en grasa originó redes proteínicas progresivamente más cerradas, que mostraron módulos viscoelásticos mayores y preferencia sensorial comparable a la del yogurt completo en grasa elaborado con sacarosa. Los yogures adicionados con CC presentaron una supervivencia de *L. plantarum* elevada (> 8.1 log cfu g^{-1}) después de 21 días de almacenamiento.

Palabras clave: yogurt, coacervado complejo, stevia, sacarosa, supervivencia de *L. plantarum*, reología, microestructura.

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1 Introduction

The demand for functional food products in which sucrose and milk-fat have been substituted by non-caloric sweeteners and fat replacers, and/or added with probiotics are on the rise (Basu *et al*., 2013; Lazaridou *et al*., 2014). On one hand, the energetic value of sucrose is considered undesirable so there is a growing interest in the food industry to use low calorie alternatives. However, the selection of an appropriate substitute is no simple matter, as sucrose provides good flavour and consistency to food products (Nip, 2007). Recently, stevia from *Stevia rebaudiana* has received increased attention for its natural origin and sweetening qualities (Basu *et al*., 2013). On the other hand, the consumption of large amounts of saturated fats in the diet is considered as a risk factor for heart disease (Lobato-Calleros *et al*., 2006). Notwithstanding, fat reduction in yogurt alters its mechanical and sensory characteristics as milk-fat globules serve as anchor points that promote protein cross-linking (Aguirre-Mandujano *et al*., 2009). The multi billion global yogurt market is a dynamic category marked by constant innovation driven by growing consumer desire for convenient and health promoting products (Research and Markets, 2015). The health benefits of yogurt can be increased by the incorporation of probiotics that contribute to improve the digestive health (Lazaridou *et al*, 2014). In spite of this, free probiotic bacteria have poor survivability in yogurt as they are liable to acid and/or aerated media (Muthukumarasamy *et al*., 2006). A common method used in the food industry for providing probiotic living cells with an increased tolerance to hostile environments, is to retain them within a biopolymer matrix acting as a physical barrier against diffusion of adverse factors (Gerez *et al*., 2012). Recently, complex coacervation has been proposed as alternative technique for microencapsulating microorganisms (Bosnea *et al*., 2014). The attractive interaction between oppositely charged biopolymers leads to the formation of soluble or insoluble complexes. A characteristic of the latter, known as complex coarcevates, is that they display superior viscoelastic properties than the individual biopolymers from which they are derived (Espinosa-Andrews *et al*., 2008). Likewise, complex coacervates have been attributed as possessing fat-mimicking functionality (Ramírez-Santiago *et al*., 2012). Hernández-Rodríguez *et al*. (2014) reported that survivability of the probiotic bacteria *L. plantarum* (Collado *et al*., 2008) was significantly increased when the cells were electrostatically bound to a whey protein isolate/κ-carrageenan complex coacervate, as compared to that of free cells after exposure to low pH and bile salts.

The objective of this work was to evaluate the effects of partially replacing milk-fat by whey protein isolate/*L. plantarum*/κ-carrageenan complex coacervate and/or sucrose by stevia on the microstructure, rheology, and sensory preference properties, and *L. plantarum* survivability of yogurt.

2 Materials and methods

2.1 Materials

The biopolymers used for the formation of the complex coacervate were whey protein isolate (WP; Hilmar TM 9400, 93 wt% protein, Hilmar Ingredients, Hilmar, CA, USA) and κ-carrageenan (KC; Grinstead® Carrageenan CH 407, Danisco Mexico, S.A. de C.V., Mexico City, Mexico). Low heat skim milk (SMP; Lactomix®, DILAC, S.A. de C.V., Mexico City, Mexico) and homogenized whole milk (WMP; NIDO®, Nestle, S.A. de C.V., Mexico City, Mexico) spray-dried powders were used to prepare the yogurt variations. Stevia or stevioside (st; 91% purity, without any carrier agents added) was used as non-caloric sweetener (Naturita Farma LDTA, Asuncion, Paraguay). Ciprofloxacin (Bayer Schering Pharma, Mexico City, Mexico) was used for differential selective growth of *L. plantarum* (Bujalance *et al*., 2006). Analytical grade hydrochloric acid (HCl) was purchased from J.T. Baker (Naucalpan, State of Mexico, Mexico). Rogosa Sharp (MRS) lactobacillus broth and agar were obtained from Becton Dickinson de Mexico, S.A. de C.V. (Mexico City, Mexico). Sucrose (su; table sugar) was purchased from a local supermarket in Mexico City. All the water used was double distilled and deionized (DDW).

2.2 Cell culture

Freeze-dried *L. plantarum* Lp-115 ATCC:SD5209 (Lp; Danisco, Braband, Denmark) was cultured for 18 h at 37 ºC (1% w/v) in sterile MRS broth under anaerobiosis (González-Olivares *et al*., 2016). The culture of Lp was sub-cultured at 37 °C for 18 h twice in sterile MRS broth using l% (w/v) of inoculums for activation and adaptation. Cells were harvested in the late logarithmic growth phase (22 h) with the help of a minispin plus Eppendorf centrifuge (Type 22331, Eppendorf AG, Hamburg, Germany) operated at 15800 × g for 10 min. The supernatant was decanted and the cells were suspended in 1 mL of physiological solution, obtaining a cell suspension containing 9.5 ± 0.1 log cfu mL^{-1}. Cell suspension was used for bacteria entrapment in the complex coacervate or as free cells in yogurt.

2.3 Complex coacervate formation

In a previous work by some of the authors of this research (Hernández-Rodríguez *et al*., 2014), it was found that complex coacervates made with a 16.7:1 WP-KC weight ratio at pH values below the isoelectric point of WP (pH ~ 4.5) showed great microstructural integrity, high viscoelastic moduli values and endowed *L. plantarum* cells with a survivability of 75.78% after sequential exposure to simulated gastric juice (pH 3.0, 37 °C, 30 min) and bile salts (37 °C, 30 min). Free cells survivability exposed to the same gastrointestinal conditions was 0.01%. Thus, the WP/Lp/KC complex coacervate was formed as indicated by Hernández-Rodríguez *et al*. (2014), with slight modifications. Briefly, WP (30 g, 5% w/v, pH 4.0, zeta potential = 3.87 ± 0.03 mV) was added with Lp cell (9.5 ± 0.1 log cfu mL^{-1}; zeta potential = -1.81 ± 0.18 mV) suspension, yielding a WP/Lp soluble complex (zeta potential = 1.53 ± 0.06 mV). Afterwards KC (9 g, 1% w/v, pH 4.0, zeta potential -30.2 ± 2.11 mV) was added using constant mild stirring (150 rpm, room temperature, 2 h). The mixture was kept at 4 °C for 48 h (zeta potential = - 3.76 ± 0.04 mV), and afterwards centrifuged at 1350 rpm for 30 min to induce complete CC precipitation. The change in zeta potential values of the mixtures clearly indicated that electrostatic interactions were the driving force for the Wp/Lp/KC complex coacervate formation.

CC structure was observed by scanning electron microscopy as described below. CC had a moisture content of 85.2 ± 1.6 wt%, and a protein content of 11.6 ± 0.3 wt% (78.2 ± 1.2 wt% d.b.). The mean volume diameter of CC was determined by dynamic light scattering measurements with a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, UK) (Hernández-Rodríguez *et al*., 2014).

2.4 Preparation of yogurt variations

Seven stirred yogurt variations were prepared in accordance to formulations given in Table 1. Two full-fat (2.6 wt% milk-fat) yogurts and two reduced-fat (1.3 wt% milk-fat) yogurts were made using as sweetener either sucrose or stevia, and coded as FFYsu, FFYst, RFYsu and RFYst, respectively. Additionally three reduced-fat yogurts were manufactured with stevia and in which milk-fat was partially replaced by CC in 1:1, 1:2 and 1:3 weight ratios, and were coded as RFYst1:1, RFYst1:2 and RFYst1:3, respectively. The substitution of sucrose was done on the basis of relative sweetening index value of stevia (300) provided by the manufacturer. Milk-fat and total milk solids contents (Table 1) of the different yogurt variations were obtained by blending WMP and SMP, and ten-liter batches of reconstituted milk were used to manufacture each one of the yogurt variations in triplicate using a completely randomized experimental design.

Table 1. Yogurt variations formulations

Yogurt variation code	Milk-fat (g 100 g^{-1})	Total milk solids (g 100 g^{-1})	Complex coacervate d.b. (g 100 g^{-1})	Sucrose (g 100 g^{-1})	Stevia (g 100 g^{-1})
FFY_{s}	2.6± 0.2	12.0 ± 0.1	-	6.0	-
FFY_{su}	2.6± 0.2	12.0 ± 0.1	-	-	0.02
RFY_{s}	1.3 ± 0.2	12.0 ± 0.1	-	6.0	-
RFY_{su}	1.3 ± 0.2	12.0 ± 0.1	-	-	0.02
$RFY_{st1:1}$	1.3 ± 0.2	10.7 ± 0.1	1.3	-	0.02
$RFY_{st1:2}$	1.3 ± 0.2	9.4 ± 0.1	2.6	-	0.02
$RFY_{st1:3}$	1.3 ± 0.2	8.1 ± 0.1	3.9	-	0.02

FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations. Subindexes su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC in dry basis (d.b.).

Batches were refrigerated at 4 ºC for 24 h to allow full hydration of powders, heated to 40 ± 1 ºC, added with the corresponding sweetening agent, pasteurized (85 ± 1 ºC, 15 min), cooled (45 ± 1 ºC) and inoculated with 0.003% w/v of freeze-dried starter culture (*Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Lactobacillus lactis*, CHOOZIT MY 800 LYO, Danisco France SAS, Dangé Saint Romain, France). Milk fermentation process was carried out at 45 ± 1 °C until an acidity of 80-85 °D was reached, determined by titration (AOAC, 1995). Afterwards the fermented milk batches were cooled and stored at 4 ± 1 °C during 24 h, and the milk gels were removed from refrigeration. At this point, FFYsu, FFYst, RFYsu, and RFYst were added with free Lp cells, while RFYst1:1, RFYst1:2, and RFYst1:3 were incorporated with CC containing the entrapped Lp cells as indicated in Table 1. All the yogurt variations were gently stirred with help of a mechanical mixer (Caframo, RZR1, Cole-Parmer, Vernon Hills, IL, USA) at 500 rpm during 1 min, and stored at 4 ± 1 °C until required for characterization.

2.5 *Chemical composition*

Yogurt variations after three days of storage were analysed for protein by the Kjeldahl method, fat by Gerber method and moisture by oven drying (AOAC, 1995). pH and acidity of the yogurt variations were determined after 3 and 21 days of storage using a Vernier pH-BTA (Beaverton, OR, USA) and titration (AOAC, 1995), respectively.

2.6 *Syneresis*

After 3 and 21 days of storage, yogurt variations (14 g) were placed in tubes and centrifuged at 222 × g for 10 min, at 4 ± 1 ºC. The clear supernatant was poured off, weighed and expressed as percent weight relative to original weight of yogurt (Keogh and O´ Kennedy, 1998).

2.7 *Survivability of L. plantarum*

One hundred g of each yogurt variation were placed into sterile glass bottles. The samples were stored at 4 °C, and the viability of Lp cells was determined during 21 days, at intervals of 7 days. One g of yogurt was placed in phosphate buffer (0.1M, pH 7.2, 2 h) to release the bound cells of Lp and cultured in MRS agar (37 °C, 48 h) added with 0.002 % w/v of ciprofloxacin (Bujalance *et al*., 2006; Sandoval-Castilla *et al*., 2010), and enumerated.

2.8 *Rheology*

Dynamic oscillatory measurements of the yogurt variations were carried out using a Physica MCR 301 rheometer (Anton Paar, Messtechnik, Stuttgart, Germany), with a cone-plate geometry, in which the rotating cone was 50 mm in diameter, and cone angle of 1° with a gap of 0.05 mm. About 3.8 mL of sample was carefully placed in the measuring system, and left to rest for 10 min for structure recovery. Amplitude sweeps were carried out to characterize the linear viscoelastic region (LVR) of the yogurt variations by applying a strain sweep ranging from 0.01 to 100% at 1 Hz. Frequency sweep test was carried out by performing a frequency ramp from 0.1 to 100 Hz (in log progression with 10 points per decade) at constant strain amplitude of 0.1% (predetermined from amplitude sweep at 1 Hz, within LVR). All the experiments were carried out at 4 °C and the temperature maintenance was achieved with Physica TEK 150P temperature control and measuring system. The storage (G´) and the loss (G´´) moduli were obtained from the equipment software (RheoPlus/32 V2.62) in all cases. Analysis was performed on each yogurt variations aged 3 days.

2.9 *Microstructure*

Microstructure of the CC and that of the yogurt variations was examined with a high vacuum scanning electron microscope Jeol JSM-035 (Jeol Ltd., Akishima, Japan) at 20 kV at different magnifications. The samples were prepared as indicated by Ramírez-Santiago *et al*. (2010).

2.10 *Sensory evaluation*

The yogurt variations aged seven days were evaluated by untrained panelists made up by 50 males and 30 females, aged between 16 and 18 years old, who were regular yogurt consumers. Each of the seven yogurt variations were placed into 20 mL plastic glasses, coded with three-digit random numbers, and randomly presented to the panelists, who were asked to score their preference for appearance, aroma, creaminess, acidity, granularity, flavour, residual flavour, and overall acceptability. Consumers' yogurt preference was scored on a five-point hedonic scale (1=dislike very much; 2=dislike moderately; 3=neither like nor dislike; 4=like moderately; 5=like very much) (Choi, 2014).

2.11 Statistical analysis

Analyses were carried out in triplicates from 3 independent experiments carried out using a randomized experimental design. Analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$) were performed on probiotic counts, chemical, syneresis, rheological and sensory data of yogurt variations using the Statgraphics 7 statistical analysis system (Statistical Graphics Corp. Manugistics Inc., Cambridge, MA, USA).

3 Results and discussion

3.1 Chemical composition

Table 2 shows the average composition of the yogurt variations. Protein content was significantly higher for all the RFY than for the FFY variations, but the opposite was observed regarding fat contents. As milk-fat:CC weight ratios increased (1:1, 1:2, and 1:3) protein contents in RFY variations increased significantly, due to the protein contribution of CC to the yogurt. Moisture content of yogurt variations containing stevia was significantly higher than that of yogurt variations made with sucrose, as the former had lower soluble solids contents (Table 1). Acidity was non-significantly different between yogurt variations aged 3 days, but increased significantly after 21 days of storage. RFY variations containing CC exhibited significantly higher acidity, probably due to the presence of the entrapped probiotic bacteria. Post-acidification during storage time can be attributed to the progressive transformation of lactose into lactic acid (Ramírez-Santiago *et al*., 2010). On the other hand, at day 3 yogurt variations showed non-significant differences in pH (4.42-4.43), but after 21 days of storage pH decreased significantly (4.01-4.22).

3.2 Syneresis

Whey separation is a major defect that may lead to consumer rejection of yogurt (Gonçalves *et al*., 2005). Although the phenomena occurring during syneresis are not fully understood, it is agreed that increased syneresis with storage time is usually associated with severe casein network rearrangements that promote whey expulsion (van Vliet *et al*., 1997). Conventionally, yogurt syneresis reduction or prevention is achieved by fortifying the protein network with dry dairy ingredients such as skim milk powder, whey protein isolate/concentrate, sodium caseinate or calcium caseinate (Amatayakul *et al*., 2006); or stabilizers such as gelatine, starch and different gums having high water binding capacity (Keogh and O'Kennedy, 1998). It is known that sucrose contributes to moisture retention in gels (Torres *et al*., 2013). In this work, syneresis of the 3 days aged yogurt variations after centrifugation at 4 ºC ranged from 5.3 to 7.5 wt% (Table 2).

Table 2. Chemical composition of yogurt variations (mean ± SD, n = 9)

Yogurt code	Moisture (wt%)	Fat (wt%)	Protein (wt%)	Syneresis 3 days (wt%)	Syneresis 21 days (wt%)	Acidity 3 days (ºD)	Acidity 21 days (ºD)
FFYsu	83.2 ± 0.4^{a}	2.6 ± 0.1^{b}	2.9 ± 0.0^{a}	5.8 ± 0.1^{abc}	7.6 ± 0.3^{ab}	84.6 ± 0.7^{a}	90.9 ± 0.4^{a}
FFYst	88.3 ± 0.5^{b}	2.6 ± 0.1^{b}	2.8 ± 0.1^{a}	5.3 ± 0.3^{a}	7.1 ± 0.1^{a}	85.4 ± 0.5^{a}	91.7 ± 0.8^{a}
RFYsu	82.7 ± 0.3^{a}	1.3 ± 0.0^{a}	3.1 ± 0.1^{b}	6.2 ± 0.2^{bc}	13.3 ± 0.6^{d}	84.2 ± 0.7^{a}	93.1 ± 0.3^{a}
RFYst	88.9 ± 0.6^{b}	1.3 ± 0.0^{a}	3.2 ± 0.1^{b}	7.5 ± 0.5^{d}	14.6 ± 0.4^{e}	83.9 ± 1.0^{a}	93.3 ± 0.2^{a}
RFYst1:1	88.7 ± 0.3^{b}	1.3 ± 0.1^{a}	3.7 ± 0.0^{c}	5.6 ± 0.6^{ab}	7.5 ± 0.4^{ab}	85.5 ± 0.6^{a}	95.5 ± 0.6^{b}
RFYst1:2	88.5 ± 0.5^{b}	1.3 ± 0.1^{a}	4.4 ± 0.0^{d}	6.7 ± 0.3^{c}	8.5 ± 0.5^{bc}	85.3 ± 0.6^{a}	104.5 ± 0.5^{c}
RFYst1:3	88.3 ± 0.6^{b}	1.3 ± 0.0^{a}	5.0 ± 0.0^{e}	7.1 ± 0.3^{d}	9.2 ± 0.4^{c}	85.8 ± 0.6^{a}	114.6 ± 0.6^{d}

FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations; su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC in dry basis. [a-e]Different superscripts within the same column indicate that means differ significantly ($p \leq 0.05$).

FFYsu, FFYst and RFYst1:1 yogurt variations displayed lowest syneresis after 3 and 21 days of storage. The rest of RFY variations showed significantly higher syneresis at all aging times. Higher water immobilization took place in FFYsu and FFYst, probably due to more numerous milk-fat globules acting as cross-linking protein agents (Lucey *et al*., 1998). The CC particles present in RFYst1:1 contributed to a water holding capacity similar to that of FFY variations. Further increases of CC in RFYst1:2 and RFYst1:3 resulted in an increased syneresis. It is known that when strong polyelectrolytes near the isoelectric point of protein associate themselves through strong intermolecular attractive forces, the resulting assemblies have lower hydration capacity than the individual biopolymers making them up (Laneuville *et al*., 2000).

3.3 Microstructure

Differences in microstructure such as association of casein micelles and porosity were qualitatively inferred from SEM micrographs. During samples preparation for SEM analysis, fat and water (whey) are removed, producing interstitial spaces between casein aggregates and minute pores in the protein structure, respectively. Thus, only the protein matrix and bacteria are visualized (Kaláb, 1993; Lee and Lucey, 2010). The SEM micrographs of the different yogurt variations are shown in Figures 1 and 2. It can be seen that substitution of sucrose by stevia and substitution of milk-fat by CC, influenced the microstructure of yogurt variations. Comparison of micrographs suggested that the microstructures of FFYsu (Fig. 1a) and RFYsu (Fig. 1b) were different. FFYsu showed a protein matrix with relatively low porosity, composed by casein micelles forming associated clusters; while RFYsu exhibited a protein matrix with increased porosity formed by smaller clusters of proteins. These results are in accordance with those of Buchheim and Dejmek (1997) who found that milk-fat globules contributed to yogurt network structuring by acting as cross-linking protein agents. FFYst (Fig. 1c) and RFYst (Fig. 1d) containing stevia exhibited matrices characterized by large, fused casein micelles clusters with comparatively lower porosity than FFYsu (Fig. 1a) and RFYsu (Fig. 1b). The presence of non-micellar material between casein clusters can also be observed in Figs. 1e and 1f. This non-micellar material appears to link the casein micelles together. Haque and Aryana (2002) informed that the type of sweetener affects the state of association of casein micelles in yogurt. Wan *et al*. (2014) reported that steviosides formed a complex with soy protein isolate, mainly through hydrophobic interactions. Ayachi *et al*. (2013) using different molecular modeling tools reported that stevioside and rebaudioside A contained in stevia extract could bind to the protein dipeptidyl peptidase-4 (DPP-4). Thus it can be hypothesized that stevia interacted with milk proteins. Increasing CC resulted in more aggregated casein micelles clusters with lower porosity (Figs. 2a-2c) than those observed in RFYst (Fig. 1d). Aziznia *et al*. (2008) reported that addition of whey protein to nonfat yogurt increased the diameter of protein particles by saturating all the binding sites of κ-casein, leading to the formation of additional whey protein aggregates. Fig. 3 shows a micrograph of CC, characterized by spherical microparticles aggregates forming an interconnected matrix, where embedded Lp cells can be observed. The mean volume diameter of the CC particles was of 231.7 ± 7.2 nm. Morris *et al*. (2000) reported that casein micelles were 100-250 nm in diameter, so that CC diameter falls within this range. Tamime *et al*. (1995) reported that fat replacer Simplesse 100 (made up by denatured whey protein microparticles) had a diameter ranging between 0.1-0.3 μm, and became an integral part of yogurt microstructure. Kaláb (1993) found that casein micelles aggregates were linked to whey protein through disulphide bridges. Patel and Velikov (2011) mentioned that the matrix of food products incorporating biopolymers, were mainly structured through non-covalent binding including hydrophobic interactions and hydrogen bonding.

3.4 Rheology

Yogurt is a viscoelastic material whose rheological properties can be described by the storage modulus (G´), which denotes its degree of elasticity, and the loss modulus (G´´), which provides a measure of its viscous nature (Guggisberg *et al*., 2011). The dependence of G´ and G´´ with frequency for yogurt variations are shown in Fig. 4. All yogurt variations exhibited a G´ characterized by showing a slight increase in gradient with frequency. G´ was always greater than G´´ over the whole frequency range studied. This behaviour is typical of entanglement networks (Peressini *et al*., 2003). For comparative purposes G´ and G´´ values were considered at 1 Hz (Table 3).

Table 3. Values of the storage (G´) and loss (G´´) moduli of yogurt variations at 1 Hz

Yogurt code	*G´* (Pa)	*G´´* (Pa)
FFYsu	112.0 ± 3.7[b]	28.5 ± 1.8[b]
RFYsu	84.8 ± 2.3[a]	21.4 ± 1.6[a]
FFYst	360.1 ± 10.0[e]	84.3 ± 5.8 [e]
RFYst	198.7 ± 10.3[c]	46.4 ± 2.3[c]
RFYst1:1	340.7 ± 9.7[d]	70.8 ± 3.0[d]
RFYst1:2	348.9 ± 10.1[d]	76.0 ± 5.7[d]
RFYst1:3	370.2 ± 11.5[e]	86.4 ± 7.4[de]

FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations. su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC in d.b. [a-e]Different superscripts within the same column indicate that mean values differ significantly ($p \leq 0.05$).

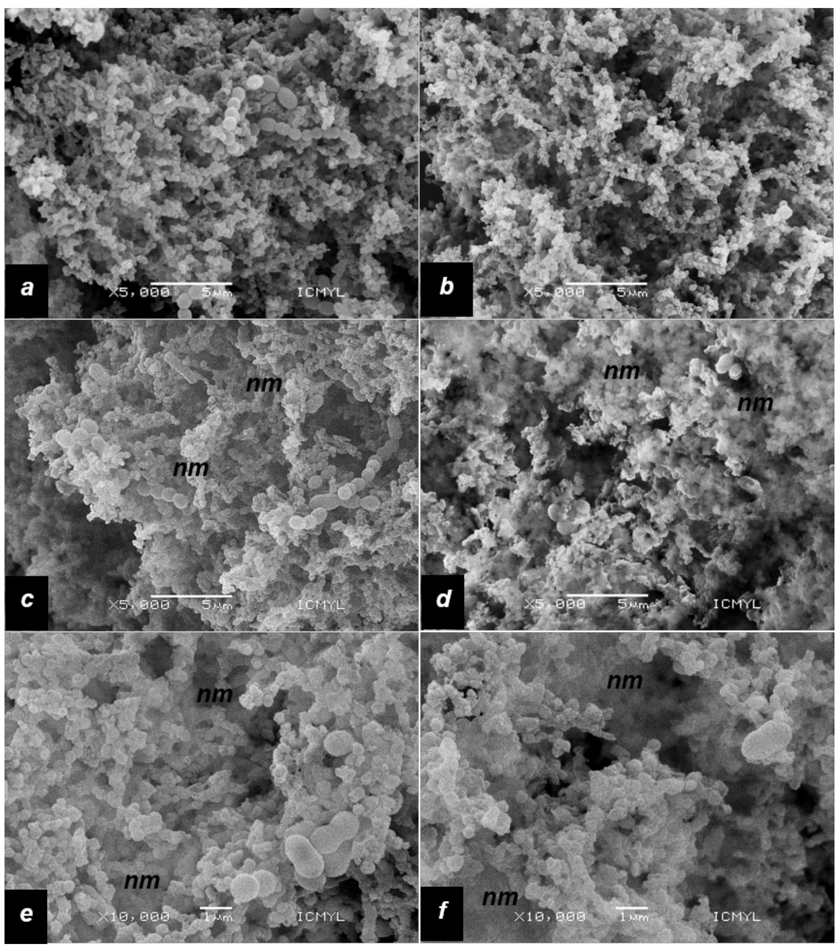

Fig. 1. SEM micrographs of full-fat yogurt with sucrose (a); reduced-fat yogurt with sucrose (b); full-fat yogurt with stevia (c, e) and reduced-fat yogurt with stevia (d, f). Non-micellar material (nm) in the protein networks of yogurts containing stevia can be observed.

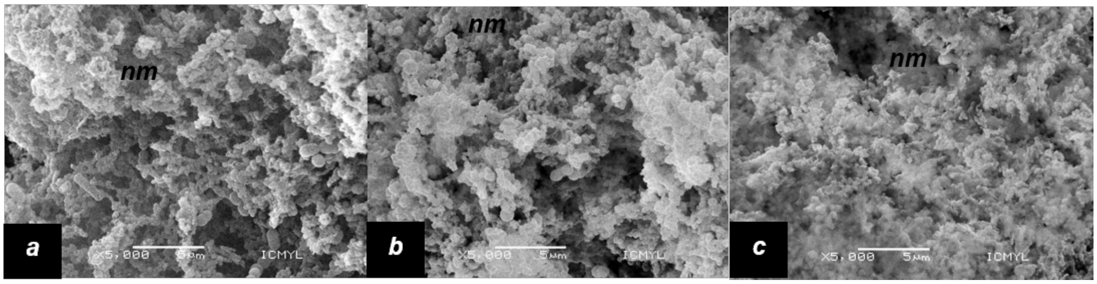

Fig. 2. SEM micrographs of reduced-fat yogurt variations made with stevia in which the complex coacervate was incorporated in 1:1 (a), 1:2 (b), and 1:3 (c) weight ratios of milk-fat to CC (d.b.). Non-micellar material (nm) in protein networks of yogurts containing stevia can be observed.

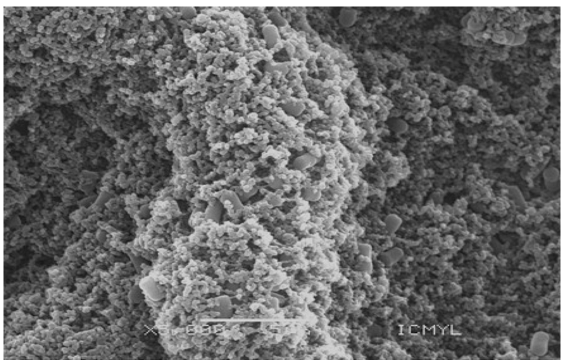

Fig. 3. SEM micrograph of the whey protein isolate/*L. plantarum*/κ-carrageenan complex coacervate.

Variance analysis confirmed that rheological parameters were significantly affected by stevia and CC inclusion in yogurt variations. The G´ and G´´ values significantly increased with the addition of stevia. Basu *et al*. (2013) reported that partial substitution of sucrose by stevioside in mango jam at certain levels led to stronger network due to hydrophobic interactions, as evidenced by FTIR spectra. Yogurts incorporating CC exhibited higher values of G´ and G´´ in comparison with those of the RFYst. These results seem to indicate probable interactions between KC and the whey proteins of CC with casein chains of the yogurt gel structure occurring via electrostatic and/or hydrophobic attractive forces, reinforcing the mechanical response of yogurt network (Baeza *et al*., 2002). It is well known that protein-polysaccharide and protein-protein interactions play a key role in the structuring and mechanical behaviour in dairy products (Corredig *et al*., 2011). It has been reported that the G´ values of gels is related to the number, strength, or both of bonds between casein particles and the spatial distribution of strands of casein in the network (Esteves *et al*., 2003). Our results indicate that the addition of stevia and CC

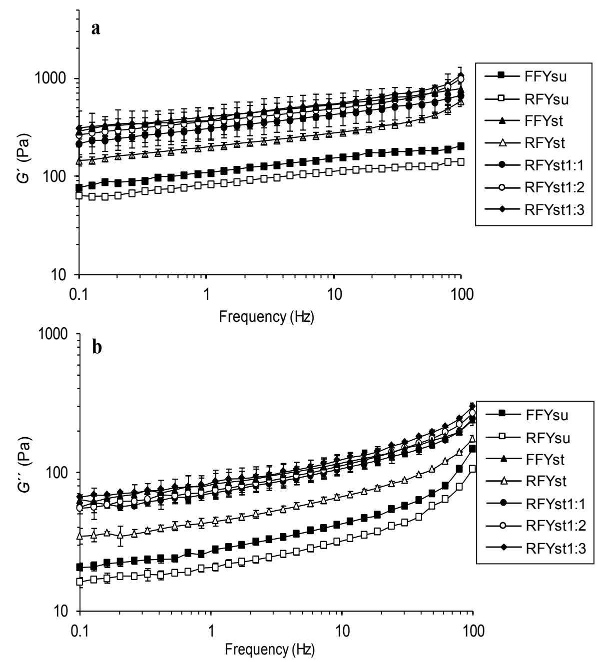

Fig. 4. Frequency dependence of the (a) storage (G´) and (b) loss (G´´) moduli of yogurt variations: (■) FFYsu; (□) RFYsu; (▲) FFYst; (△) RFYst; () RFYst1:1; (o) RFYst1:2; and (♦) RFYst1:3. FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations. su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC (d.b.).

to reduced-fat yogurts contributed to the gels formation with an increased viscoelastic behaviour, compared to the controls (FFYsu and RFYsu). There was a relationship between the microstructure of yogurt and viscoelastic moduli. Yogurts which showed a denser structure and lower porosity exhibited higher G´ and G´´ values.

Table 4. Viability of *Lactobacillus plantarum* in yogurt variations during storage

Yogurt code	log cfu g^{-1}			
	day 1	day 7	day 14	day 21
FFYsu	$8.25 \pm 0.1^{a,D}$	$7.56 \pm 0.1^{a,C}$	$6.83 \pm 0.1^{a,B}$	$6.53 \pm 0.1^{a,A}$
FFYst	$8.20 \pm 0.1^{a,D}$	$7.51 \pm 0.1^{a,C}$	$6.80 \pm 0.1^{a,B}$	$6.53 \pm 0.1^{a,A}$
RFYsu	$8.27 \pm 0.2^{a,D}$	$7.57 \pm 0.1^{a,C}$	$7.24 \pm 0.1^{b,B}$	$6.57 \pm 0.1^{a,A}$
RFYst	$8.23 \pm 0.2^{a,D}$	$7.55 \pm 0.1^{a,C}$	$6.81 \pm 0.1^{a,B}$	$6.53 \pm 0.1^{a,A}$
RFYst1:1	$8.11 \pm 0.2^{a,A}$	$8.07 \pm 0.1^{b,A}$	$8.09 \pm 0.1^{c,A}$	$8.13 \pm 0.1^{b,A}$
RFYst1:2	$8.19 \pm 0.1^{a,A}$	$8.14 \pm 0.2^{bc,A}$	$8.26 \pm 0.1^{d,A}$	$8.27 \pm 0.1^{bc,A}$
RFYst1:3	$8.12 \pm 0.1^{a,A}$	$8.32 \pm 0.1^{c,B}$	$8.34 \pm 0.1^{d,B}$	$8.40 \pm 0.1^{c,B}$

FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations; su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC in d.b. $^{a-d}$Different superscripts within the same column indicate that mean values differ significantly ($p \leq 0.05$). $^{A-D}$Different superscripts within the same row indicate that mean values differ significantly ($p \leq 0.05$).

3.5 Survival of *L. plantarum* in yogurt

Initial counts of free and entrapped *L. plantarum* cells in all yogurt variations were non-significantly different (Table 4). However, at the end of the refrigerated storage time (21 days), entrapped *L. plantarum* cells within CC did not suffer loss of viability, while free cells presented high viability losses (Table 4). Cell counts of RFYst1:1, RFYst1:2, and RFYst1:3 remained above 10^8 cfu.g^{-1}, complying with the recommended minimum numbers of 10^7 cfu g^{-1} of live cells at the time of consumption, to be considered as probiotic food product (Ferdousi *et al*., 2013), while free cells contained in FFYsu, FFYst, RFYsu and RFYst variations did not. During processing and storage of foods, probiotic microorganisms can suffer viability losses.

In the particular case of yogurt, exposition to high acidity, low pH, high osmotic pressure and high contents of oxygen, lactic and acetic acids have been identified as having an effect on probiotics viability during manufacture and storage of yogurt (Dave and Shah, 1997; Ayama *et al*., 2014). Shah and Jelen (1990) stated that the main factor affecting the survival of probiotic bacteria in yogurt is the increasing acid content during fermentation and storage. As can be seen in Table 2, the acidity of all yogurt variations increased with storage time. In spite of RFY's containing CC exhibited the highest acidity values, *L. plantarum* did not show viability losses. In a previous work it was found that complex coacervate obtained from the interaction between WP/Lp/KC provided an adequate protection to *L. plantarum* cells when they were exposed to simulated gastric pH of 3.0 (Hernández-Rodríguez *et al*., 2014). It is clearly seen in Fig. 3 that the Lp cells were immobilized within CC biopolymer matrix. The CC biopolymer matrix could afford protection to the cells by slowing down the diffusion rate of compounds produced during fermentation and storage of yogurt such as acids and hydrogen peroxide (Sandoval-Castilla *et al*., 2010). Brusch-Brinques and Záchia-Ayub (2011) found that immobilization of *L. plantarum* in different biopolymer matrices increased cells survivability in yogurt under refrigerated storage. Shoji *et al*. (2013) reported that *L. acidophilus* encapsulated by complex coacervation and incorporated into buffalo milk yogurt presented greater stability compared to the yogurt prepared with the free culture.

3.6 Sensory evaluation

The market acceptance of novel foods is driven by consumer's choice, and thus appearance, flavour, taste and mouthfeel become critically important factors. Colloidal delivery systems need to be designed in a way that they improve, or at least do not diminish, the overall acceptability of the product (Patel and Velikov, 2011). Table 5 shows the sensory evaluation scores of the yogurt variations.

Table 5. Mean sensory attributes and overall acceptability scores of yogurt variations

Yogurt code	Aroma	Appearance	Creaminess	Acidity	Granularity	Flavour	Residual flavour	Overall acceptability
FFYsu	4.1 ± 1.2^{b}	3.9 ± 1.5^{a}	4.0 ± 1.2^{ab}	3.9 ± 1.2^{b}	4.3 ± 1.1^{b}	4.5 ± 0.9^{b}	4.0 ± 1.3^{b}	4.2 ± 1.2^{b}
FFYst	4.0 ± 1.1^{b}	3.8 ± 1.2^{a}	3.9 ± 1.2^{ab}	3.8 ± 1.2^{b}	4.2 ± 1.1^{b}	4.3 ± 1.0^{b}	4.3 ± 1.0^{b}	4.0 ± 1.1^{b}
RFYsu	3.7 ± 0.3^{a}	3.6 ± 1.5^{a}	3.3 ± 1.3^{a}	2.9 ± 1.4^{a}	3.4 ± 1.8^{a}	2.1 ± 1.5^{a}	3.9 ± 1.0^{a}	2.4 ± 1.5^{a}
RFYst	3.6 ± 1.2^{a}	3.7 ± 1.2^{a}	3.3 ± 1.3^{a}	3.3 ± 1.4^{a}	3.5 ± 1.8^{a}	2.3 ± 0.9^{a}	3.7 ± 1.3^{a}	2.3 ± 1.3^{a}
RFYst1:1	4.4 ± 1.0^{b}	4.0 ± 1.2^{a}	4.8 ± 0.6^{c}	4.2 ± 1.2^{b}	4.0 ± 1.4^{ab}	4.7 ± 0.7^{b}	4.2 ± 1.2^{b}	4.6 ± 0.8^{b}
RFYst1:2	4.0 ± 1.3^{b}	4.0 ± 1.4^{a}	4.2 ± 1.1^{bc}	3.8 ± 1.2^{b}	4.2 ± 1.2^{ab}	4.2 ± 1.2^{b}	4.0 ± 1.4^{b}	4.1 ± 1.3^{b}
RFYst1:3	4.0 ± 1.5^{b}	3.7 ± 1.3^{a}	4.4 ± 1.0^{bc}	4.1 ± 1.4^{b}	3.9 ± 1.5^{ab}	4.4 ± 1.0^{b}	4.0 ± 1.4^{b}	4.3 ± 1.1^{b}

FFY: full-fat yogurt variations; RFY: reduced-fat yogurt variations; su = sucrose; st = stevia; 1:1, 1:2 and 1:3 = weight ratios of milk-fat to CC in d.b. $^{a\text{-}c}$Different superscripts within the same column indicate that mean values differ significantly ($p \leq 0.05$).

RFY made with sucrose and stevia exhibited in general lower sensory attributes scores than their FFY counterparts, with the exception of appearance. The partial or total removal of fat from yogurt decreases the overall quality perceived by the consumer due to changes in texture and in the retention of flavour compounds in the product, as also fat has its own aroma and flavour (Cayot *et al.*, 2008). On the other hand, the RFY variations made with stevia + CC showed comparable aroma, acidity, granularity, flavour, residual flavour, and overall acceptability sensory scores than the FFYsu. In this manner, it is assumed that the combination of stevia + CC displayed milk - fat mimetic functionalities, yielding reduced milk-fat yogurts with comparable sensory attributes as those of a full-milk fat yogurt made with sucrose.

Conclusions

In this work it was demonstrated that the molecular features and concentration of the added ingredients (whey protein isolate/*L. plantarum*/κ-carrageenan complex coacervate, and stevia) affected significantly the gel strength (microstructural arrangement and rheological properties) of the yogurt variations. The combination of stevia and the complex coacervate yielded reduced milk-fat yogurts, whose microstructure was composed by spherical microparticles aggregates forming an interconnected matrix, exhibiting higher viscoelastic behaviour and comparable sensory attributes as those of a full-milk fat yogurt made with sucrose. SEM micrographs clearly show that *L. plantarum* cells were immobilized within the complex coacervate matrix. Immobilized probiotic cells survivability was significantly higher than free cells survivability in yogurt, thus it is postulated that the complex coacervate matrix afforded an effective protection to the cells by acting as a physical barrier against adverse environmental conditions.

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