Multiscale design of a dairy beverage model composed of *Candida utilis* single cell protein supplemented with oleic acid

H. M. Buitrago Mora,¹ M. A. Piñeros,¹ D. Espinosa Moreno,¹ S. Restrepo Restrepo,² J. E. C. Cardona Jaramillo,¹ O. A. Álvarez Solano,¹ M. Fernandez-Niño,¹ and A. F. González Barrios^{1*}

¹Grupo de Diseño de Productos y Procesos, Department of Chemical Engineering, Universidad de los Andes, Bogotá 111711, Colombia

²Vice-rectory of Research and Laboratorio de Micología y Fitopatología, Biological Sciences Department, Universidad de Los Andes, Bogotá 111711, Colombia

ABSTRACT

One of the main challenges in the food industry is to design strategies for the successful incorporation of natural sources of bioactive compounds. Recently, yogurts and other fermented dairy beverages have been proposed as ideal carriers of such bioactive compounds such as fatty acids and antioxidants that could improve consumers' health. However, the incorporation of new ingredients causes functional and structural modifications that may affect the consumers' preferences. In this work, a dairy beverage model supplemented with oleic acid has been designed by partial substitution of milk by *Candida utilis* single-cell protein extract. The changes in the structural properties of this new beverage were evaluated by following the fermentation process, pH, aggregate size, microstructure, and changes in rheological properties. Furthermore, molecular dynamics simulations were carried out to analyze the interaction between its main components. Our data revealed that samples with a percentage of milk substitution of 30% showed a higher viscosity as compared with the other percentages and less viscosity than the control (no substitution). These samples were then selected for fortification by incorporating oleic acid microcapsules. A concentration of 1.5 g/100 g was shown to be the optimal quantity of microcapsules for oleic acid supplementation. Molecular dynamic simulations revealed glutathione as an important component of the microgel structure. The present study forms the basis for novel studies where *Candida utilis* single-cell protein and microencapsulated essential oils could be used to design innovative bioproducts.

Key words: fermented dairy beverage, single cell protein, *Candida utilis*, microencapsulation, multiscale design

INTRODUCTION

Fermented dairy beverages (FDB) are among the most consumed milk products in the world (Sakin-Yilmazer et al., 2014; Pandalaneni et al., 2018; Cordeiro et al., 2019). In addition to their high nutritional value, FDB have been associated with many health benefits because of their microbial composition and functional metabolites (Macedo and Vélez-Ruiz, 2015; Pelaes et al., 2015; Vardjan et al., 2018; Aguilar-Raymundo and Vélez-Ruiz, 2019). Today, FDB have become an excellent matrix to deliver bioactive compounds that might enhance people's health and reduce the risk of some diseases (Macedo and Vélez-Ruiz, 2015). In this regard, a large number of fermented dairy beverages have been designed with the addition of bioactive compounds to deliver extra health benefits over their basic nutritional value (Chouchouli et al., 2013; Pelaes et al., 2015; Barkallah et al., 2017; Öztürk et al., 2018; Aguilar-Raymundo and Vélez-Ruiz, 2019). Many of these additives such as n-3, *Nigella sativa* seed oil, echium oil, marine fish oil, astaxanthin from *Phaffia rhodozyma*, and β -carotene have been microencapsulated prior to addition to delay their degradation (Estrada et al., 2011; Toniazzi et al., 2014; Abedi et al., 2016; Comunian et al., 2017; Feng et al., 2018). One of the most interesting functional components that could be incorporated into an FDB is oleic acid (OA). This MUFA has been associated with several health benefits, such as a reduction in low-density lipoprotein cholesterol levels and the total cholesterol/high-density lipoprotein-C ratio (FAO and FINUT, 2012). In addition, supplementation with 0.28 mg of OA in Swiss-Webster mice diet has been shown to prevent organ dysfunction and mortality in experimental sepsis (Gonçalves-de-Albuquerque et al., 2016). Interestingly, a 4% OA supplementation in the total calories consumed by Wistar mice showed an antioxidant effect, generating a neurodegenerative oxidation effect in a Huntington's disease experimental model (Alconchel-Gago et al., 2014). However, given that OA is susceptible to oxidation by oxygen or light, it might

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*Corresponding author: andgonza@uniandes.edu.co

be necessary to use innovative technics to improve its thermostability, as well as maintain its functional and biological activity (López Hernández, 2010; Bakry et al., 2016). Consequently, one of the main challenges in the beverage industry is to develop strategies for the successful incorporation of natural sources of bioactive compounds in FDB such as fatty acids and single-cell protein (SCP).

The yeast *Candida utilis* is an excellent platform for the production of SCP for animal and human feeding (Buerth et al., 2016). This yeast has an important nutritional value, possessing a high protein content (55%) and an interesting AA profile, which includes lysine (4.5%), threonine (3.0%), histidine (2.0%), and arginine (4.9%), as well as B-complex vitamins (Lucca et al., 1995). Moreover, *C. utilis* has endogenous bioactive compounds such as glutathione, glucomannan, biotin, and L-phenylacetylcarbinol (Buerth et al., 2011a; Tomita et al., 2012), thus becoming an interesting natural source of both protein and bioactive compounds. Nevertheless, no studies have been reported in which *C. utilis* or its extract have been incorporated in FDB.

The incorporation of new ingredients in FDB causes functional and structural modifications, thus affecting consumers' preferences (Pelaes et al., 2015; Vardjan et al., 2018; Aguilar-Raymundo and Vélez-Ruiz, 2019). These changes in macroscopic properties are related to the microstructure that is formed by suspended solids, the aggregate formation profile in gel transformation, and the exopolysaccharides from bacteria (Rodríguez et al., 2015; Vardjan et al., 2018). The aggregate formation and microstructure depend on temperature, pH reduction, and other aspects not yet elucidated (Lee and Lucey, 2010). At the molecular level, milk caseins interact with one another and with the new molecules incorporated, where κ -casein plays an important role in acid gelation (Dalgleish and Corredig, 2012). The aim of this work was to study the effect of partial substitution of milk by *Candida utilis* SCP extract and the subsequent supplementation with microencapsulated OA using a multiscale approach (Charpentier, 2002; Pradilla et al., 2015).

MATERIALS AND METHODS

Strain and Growth Conditions

Candida utilis ATCC 9950 (Kwik-Stik, Microbiologics Inc., St. Cloud, MN) was routinely cultivated on solid potato dextrose agar medium for 48 h at 30°C, following the streak-plating method. Cells from a single colony were then used to inoculate 500 mL of liquid yeast extract-peptone-dextrose (YPD) medium, containing 10 g/L of yeast extract, 20 g/L of peptone,

and 20 g/L of dextrose. Cultures were subsequently incubated in an orbital shaker at 250 rpm and 30°C for 16 h. Optical densities (OD) were determined at 473 nm using a Genesys 10s UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA) until an OD of 2.13 was reached. This culture was then used as inoculum for batch fermentations.

Batch fermentations were carried out using a BioFlo/CelliGen 115 bioreactor (New Brunswick, Germany), containing 4.5 L of YPD medium. Fermentations were carried out at 30°C, with an aeration rate of 1 L/min and continuous agitation for 8 h at 300 rpm, until an OD = 1.82 was reached. This fermented broth was then transferred into plastic bottles, centrifuged at $1,400 \times g$ for 30 min at 16°C, recovered in 50-mL plastic tubes and finally centrifuged at $1,400 \times g$ for 15 min at 16°C. The obtained pellet was washed 3 times with ultrapure water (Direct-Q 3UV-R, Merck-Millipore, Burlington, MA), thus obtaining the SCP biomass.

Biomass Characterization and Protein Extraction

Moisture (AOAC 934.01; AOAC International, 1995) and total nitrogen Kjeldahl (SSSA series 5) (Bremner, 1996) were determined for SCP biomass. Protein content in SCP biomass was determined by multiplying the total nitrogen value by a nitrogen conversion factor of 6.25, as described by Øverland et al. (2013) and expressed on a dry basis (g/100 g). To use SCP biomass as a protein source in beverage fermentations, an initial lysis step by thermic treatment (−20 to 50°C) was required, followed by separation of the cellular membrane through centrifugation at $1,400 \times g$ for 10 min. The obtained single-cell protein extract (SCPE) was diluted with deionized water to obtain a concentration of 3.2 g/100 mL, and the total protein content was then estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Beverage Fermentation Procedure

Dairy single-cell protein extract beverages (DAS-CPEB) were prepared using skimmed and lactose-free UHT milk (Alpina, Colombia; i.e., 3.2 g/100 mL of protein and 8.2 g/100 mL of DM) and the effects of milk substitution with SCPE in beverage fermentations were subsequently determined. A dairy beverage without substitution was used as control (0% substitution) and experiments were carried out by mixing SCPE with milk in fermentation flasks in the following proportions: 10, 20, 30, 40, 50, and 60 mL/100 mL. Then, DASCPEB non-fatty solids were standardized to 8.2 g/L using sugar (Manuelita, Cali, Valle del Cauca, Colombia) and subsequently homogenized. After that, the

different mixes prepared and the control samples were inoculated with 0.1 g/100 mL of commercial lyophilized starter culture containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* (*Lactobacillus bulgaricus*), and *Bifidobacterium* (Centro Agrolechero, Bogotá, Colombia). Finally, samples were fermented by incubation at 42°C for 6 h and stopped by cooling. At least 2 biological replicates of each experiment were carried out.

Oil in Water Emulsions

Before OA microencapsulation, 5 emulsions were prepared according to Di Battista et al. (2015) with some modifications. Initially, Arabic gum (15 g/100 g) and maltodextrin (5 g/100 g) were dissolved in water and heated at 50°C for complete dissolution (mix-1). Then, OA (5 g/100 g) and Tween 20 were added in different ratios (i.e., 1, 2, 3, 4, and 5 g/100 g) until dissolution was observed (mix-2). Mix-1 was cooled at 25°C and homogenized at 3,000 rpm using a Dissolver LC75 DISPERMAT (VMA, Reichshof, Germany) and mix-2 was added drop by drop to it, allowing emulsion formation. The obtained mix (mix-3) was further homogenized for 20 min. Finally, each emulsion was homogenized for 9 min at 24,900 rpm, using a Micra D-15 homogenizer (Micra, Heitersheim, Germany).

Emulsion stability was determined by scan transmission and backscattering using a Turbiscan LAB Stability Analyzer (Formulaction SA, Toulouse, France). A total of 15 mL of each emulsion was used for this analysis, for a time period of 45 min. Turbiscan stability index was determined. Drop formation was evaluated using a BA301 optical microscope (Motic, Hong Kong), while its distribution was determined by dynamic light scattering (DLS) using a Mastersizer 3000 equipment (Malvern Instruments, Malvern, UK). The Brouckere mean diameter $D[4,3]$ was determined for samples diluted in deionized water, displaying 10.5% of obscurity. Finally, the most stable emulsion (i.e., 4% surfactant emulsion) was selected and total suspended solids (total feed solids) were measured before drying using a 30033 refractometer (Sper Scientific, Scottsdale, AZ).

Oleic Acid Microencapsulation—Spray-Drying

Initially, the obtained emulsion was dried using a Mini Spray Dryer (B-290, Büchi Labortechnik AG, Flawil, Switzerland) provided with a 0.7-mm diameter nozzle. Operating conditions included setting the following conditions: drying air inlet temperature at 160°C, atomization air in-flow rate at 601 L_{air}/h, feed volumetric flow rate at 4 mL/min, and drying air volumetric flow rate at 35 m³/h. Process yield (PY) was

assessed according to Di Battista et al. (2015) using the following equation:

$$PY = [(\text{powder weight obtained}) / (\text{TS fed})] \times 100\%. \quad [1]$$

Microencapsulation efficiency (MEE) was calculated using equation 2, as previously reported by (Jiménez-Martín et al., 2014). Briefly, the total OA amount (TOA) was determined using 2 g of microcapsules, where the total oily matter was recovered using a Soxhlet extraction system with n-hexane during 4 h by rotary evaporation, and then it was weighted. For free OA amount (OAF), 2 g of microcapsules was diluted in 20 mL of ethyl ether, heated to 50°C, and filtered initially with an 11-mm filter, and subsequently, using a 0.22-μm filter. Then, this solvent was evaporated using a rotary evaporator and the remaining oil matter was weighted afterward.

$$MEE = (\text{TOA} - \text{OAF} / \text{TOA}) \times 100\%. \quad [2]$$

Microcapsule morphology was then evaluated using a Phenom Pro X Scanning Electron Microscope (Phenom-World, Eindhoven, the Netherlands). Microcapsules were initially fixed on a stub cover with carbon tape and sputtered with gold. Images were visualized at 10 kV and recorded. The microcapsule size distribution was measured by DLS using a Mastersizer 3000 equipment (Malvern Instruments), dissolving the powder in 500 mL of deionized water until 10.5% obscurity was obtained, and then the Brouckere mean diameter $D[4,3]$ was determined.

Microcapsule Supplementation Containing Oleic Acid

The DASCPEB in a ratio of 30 mL/100 mL was selected as the upper limit substitute for microcapsule supplementation and was subsequently used to assess the supplementation effect on the beverage properties. Three independent DASCPEB (all of them in a ratio of 30 mL/100 mL) were fermented, stirred, and further supplemented with microcapsules at concentrations of S-A = 1.5, S-B = 3, and S-C = 4.5 g/100 g, respectively. Supplementation was initially carried out in 10% of its original volume to avoid changes on viscosity. Then, the volume was completed with the remaining DASCPEB volume. The DASCPEB in a ratio of 30 mL/100 mL without supplementation was used as the experimental control. At least 2 biological replicates of each experiment were carried out. Samples were stored at 4°C to further evaluate their physicochemical properties.

Physicochemical Determinations

pH was measured each hour in the substitution experiment and at 1, 8, 15, and 21 d for the supplementation experiment, using a Seven-Compact pH-meter (Mettler Toledo, Columbus, OH). Additionally, the physical properties cited below were measured at the same time intervals as pH.

Rheological Behavior. Rheological properties of beverages were characterized using a Discovery HR-1 rheometer (TA Instruments, New Castle, DE), equipped with a concentric geometry cylinder (Deutsches Institut für Normung, 15 mm radius and 42 mm height) and Peltier temperature control that kept the samples at 20°C. An aliquot of 20 mL of each sample was loaded in the cylinder, pre-sheared for 60 s at 1 s⁻¹, and followed by a back and forth sweep probe, from 1 to 500 s⁻¹ shear rates.

Average Aggregate Size. The average aggregate size as a function of pH and storage time was determined according to Rodriguez et al. (2013) by DLS using a Mastersizer 3000 equipment (Malvern Instruments), maintaining 10.5 to 11.0% of obscuration. Measurements were recorded 5 times for each sample and the Sauter mean diameter (D[3,2]) average was determined.

Microstructure. The obtained beverage gel microstructure was observed using a Phenom Pro X Scanning Electron Microscope (Phenom-World). Initially, an aliquot of 5 µL of each sample was placed on a stub cover with carbon tape, then dried in a convection oven at 25°C, and subsequently sputtered with gold covering. Finally, samples were observed at 10 kV and micrographs were obtained. For the substitution assay, micrographs were taken at the start and the end of the fermentation, whereas for the supplementation assay, they were taken on d 1 and after 21 d.

Molecular Dynamics Simulations

In silico assays were carried out using molecular dynamics (MD) to study the glutathione interactions with *C. utilis* extracts, microcapsule components, and milk proteins. First, we selected the κ-casein as the protein expected to interact with other components in dairy beverage formation and glutathione as the bioactive component from the extract. The κ-casein AA sequence (accession number: AAQ87923.1) was downloaded from the National Center for Biotechnology Information web page. The tertiary structure of kappa casein was predicted by homology using the I-TASSER (Iterative Threading ASSEMBly Refinement) protocol (Zhang, 2008; Roy et al., 2010; Yang et al., 2015) with a C-score of -4.13. Then, maltodextrin,

OA, and glutathione 3-dimensional structures were downloaded from PubChem (Kim et al., 2016). Arabic gum 3-dimensional structure was drawn according to Ikram and Ahnmed (2016) in Avogadro 1.2 (an open-source molecular builder and visualization tool), and the energy minimization structure was carried using universal force field in 5,000 cycles of steepest descent. Topologies for small molecules were then generated using SwissParam (Zoete et al., 2011) and the protein topology was generated with Intermol (Shirts et al., 2017). Next, the fourth cubic simulation box was created with 11 nm³ and periodic boundaries for (a) free κ-casein, (b) solvated κ-casein, (c) solvated κ-casein and glutathione, and (d) κ-casein solvated with glutathione, Arabic gum, maltodextrin, and OA.

Molecular dynamics simulations were run using LAMMPS version 11-Aug-17 (<http://lammps.sandia.gov>; Plimpton, 1995) into an Ubuntu virtual machine using a hybrid inter-harmonical potential (harmonic and CHARM) and applying the following script code steps: (1) one minimization on steepest descent followed by one on a conjugate gradient, and (2) an equilibration during 0.35 ns in an NVE system. Finally, the simulation by 1 ns with a Berendsen thermostat to 298 K in an NPT (number of particles, pressure, and temperature) system was done. The trajectory and the results were visualized in Ovito (<http://ovito.org>; Stukowski, 2010).

RESULTS AND DISCUSSION

SCP Biomass and SCP Extracts Were Obtained from Batch Fermentations of *C. utilis*

Batch fermentations of *C. utilis* were initially performed to obtain SCP biomass. Biomass of 3.36 g/L on a dry basis was obtained, which agrees with that reported in the literature where *C. utilis* net biomasses ranged from 2.9 to 9.80 g/L (Tobajas et al., 2003; García et al., 2014; Prakash and Mohamed, 2017). These values have been also shown to be affected by medium composition and operating conditions (Tobajas et al., 2003; García et al., 2014; Prakash and Mohamed, 2017). The total protein and moisture content in SCP biomass were then determined following the standard protocols described in the Materials and Methods section. The total protein content of 40.02 g/100 g on a dry basis and moisture content of 71.99 g/100 g were obtained.

The protein value reported here was lower than that frequently reported in the literature (i.e., ranging from 43.24 to 49.95 g/100 g), which can be explained by the fact that a different carbon source was used and experiments were conducted without supplementation

Figure 1. pH profiles for dairy single-cell protein extract (SCPE) beverages during fermentation. Experiments were carried out by mixing SCPE with milk in fermentation flasks in the following proportions: 0 (control), 10, 20, 30, 40, 50, and 60 mL/100 mL (shown here as substitution percentage). Graphics show each pH profile per sample, taken each hour during 6 h of fermentation. Error bars represent SD. The horizontal line indicates the isoelectric point of casein.

(Carrillo et al., 2010; Buerth et al., 2011b; Rodríguez et al., 2011). Finally, an extract of *C. utilis* SCP was obtained by thermic treatment and separation of the cellular membrane. The total protein content in this extract was determined to show a value of 3.26 ± 0.08 g/100 g.

SCPE Substitution Seems to Differentially Affect the pH During DASCPEB Fermentations

The pH of DASCPEB decreased through fermentations in response to lactic acid production during bacteria growth. Our results suggest that the percentage of SCPE substitution seems to differentially affect the pH through the fermentation (Figure 1). This effect can be related to the fiber present in the extract (Puvanenthiran et al., 2014; Pelaes et al., 2015). Lower percentages of SCPE substitution (i.e., 10, 20, and 30 mL/100 mL) displayed a behavior comparable to the control (i.e., 0% substitution). In contrast, higher percentages of substitution (40, 50, and 60 mL/100 mL) showed a steeper decrease, especially after 4 h of fermentation

(Figure 1). These samples were shown to exceed the isoelectric point of caseins ($\text{pH} = 4.6$) after 6 h of fermentation. Thus, samples were divided into 2 groups according to their substitution percentage: low (10, 20, and 30 mL/100 mL) and high substitution (40, 50, and 60 mL/100 mL).

DASCPEB Fluid Behavior Was Affected According to the Amount of Milk Substituted by SCPE

The fluid behavior and apparent viscosity of DASCPEB were measured using a rheometer. All DASCPEB showed an apparent decrease in viscosity, while the shear rate increased, which resembles a typical pseudo-plastic behavior (Figure 2) and follows the power law, $\sigma = k(\gamma)^n$, where σ is the shear stress, k is the consistency index (apparent viscosity), γ is the shear rate, and n is the fluid behavior.

An increase in apparent viscosity was shown to vary in response to the fermentation time and SCPE concentration. The apparent viscosity of DASCPEB was shown to remain constant during the first 1 to 3 h

of fermentation (Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-16729>), continuously increase after 4 h, and finally decrease after 6 h (at the end of the fermentation; Figure 3). It is important to mention that an increase in viscosity was directly observed during the first hours of fermentation for the sample with a 50% substitution, which could be associated with the rapid pH drop analyzed previously for this sample, whereas the other samples remained constant during this time.

The sample that showed the highest increase in apparent viscosity after 6 h (i.e., 1.230 ± 0.022 Pa·s) was that with a 30% of substitution, which agrees with the reported by Castro et al. (2013), who also observed a high apparent viscosity (i.e., 0.821 ± 0.030 Pa·s) in samples where milk was substituted by whey. Nevertheless, it is important to mention that after 6 h all samples showed a lower apparent viscosity as compared with the control (Figure 3), which is concomitant with the fact that pH decreased after 4 h.

Viscosity is one of the most important consumer characteristics in dairy products and is affected by the solids volume suspended in a liquid, the starter culture, and the fortification, among other things (Lee and Lucey, 2010; Rodríguez et al., 2015). When the beverage

was fortified with natural sources as *Spirulina platensis* powder or *Pleurotus ostreatus* extract, the beverage stiffness decreased and the viscosity was affected in a positive or negative way (Pelaes et al., 2015; Barkallah et al., 2017). To carry out a rational beverage design and understand what happens when a natural bioactive source is incorporated, it is also important to understand the casein aggregation profile, the microstructure, and the relevant molecular interactions involved.

A Low Amount of Milk Substituted by SCPE Exhibited a Similar Aggregation Profile to a Dairy Beverage

The DLS technique was reported to obtain the aggregation profiles (size increase) as a function of pH and time in beverage fermentation (Lee and Lucey, 2010; Rodríguez et al., 2015). The control sample showed an aggregation profile in accordance with previous studies (Figure 4A). Briefly, for pH values between 6.7 and 6, no increase was shown. After that, the aggregation profile exhibited 2 inflection points at pH 6.0 and 5.0, the first corresponding to a net charge decrease, an increase in the steric stability, and a rise in the colloidal calcium

Figure 2. Fluid behavior for dairy single-cell protein extract (SCPE) beverages in a shear rate from 0 to 500 s^{-1} . Trends show the pseudoplastic behavior for each sample, and as it increases, this behavior decreases. Experiments were carried out by mixing SCPE with milk in fermentation flasks in the following proportions: 0 (control), 10, 20, 30, 40, 50, and 60 mL/100 mL (shown here as substitution percentage).

Figure 3. Apparent viscosity (k value) for each treatment. The graphic shows the apparent viscosity for each treatment after 6 h of fermentation. Experiments were carried out by mixing single-cell protein extract with milk in fermentation flasks in the following proportions: 0 (control), 10, 20, 30, 40, 50, and 60 mL/100 mL (shown here as substitution percentage). Error bars represent SD.

phosphate dissolution rate, and consequently, an increase in the aggregate size. Second, at a pH lower than 5.0 all the colloidal calcium phosphate is lost, favoring hydrophobic and short-range interactions to cause the sol-gel formation (Lee and Lucey, 2010; Dalglish and Corredig, 2012). Therefore, the DLS measurements can be used to identify trends for DASCPEB aggregation profiles.

All of the treatments showed a particle size increase after 3 fermentation hours, an early increase (2 h) for the sample at 50%, and any change exhibited at 60% (Supplemental Figure S2, <https://doi.org/10.3168/jds.2019-16729>; Figure 4B and C), which explain the behavior described above regarding the viscosity. The 10% DASCPEB presented a delay in the particle size increase (pH 5.5; Figure 4B); meanwhile, samples at 20 and 30% present a slight acceleration effect (in the 3rd hour) and increased particle sizes at pH 6.15 and 6.64, respectively. Besides, the sample at 30% for 5 h achieved a particle size similar to the control sample (12.6 μm) at 7 h later (data not shown in Figure 4A). A high substitution percent exhibited a different particular trend (Figure 4C): at a pH of 5.5, the sample at 40% had the same increase in particle size as the sample at 10%; the only difference is that the first one obtained this increased particle size 1 h before. On the other hand, samples at 50 and 60% showed a disorganized aggregation. The DASCPEB kinetics of protein aggregation play an important role and were directly associated with the viscosity obtained. As it is well known for dairy beverage formation, pH and temperature have a specific effect on forming protein-protein bonds, and in

consequence, cause a rearrangement of the protein clusters and microstructure formation. This microstructure influences viscosity (Lee and Lucey, 2010; Rodríguez et al., 2015).

A Low Amount of Milk Substituted by SCPE Displayed a Similar Microstructure as Dairy Beverage

The casein 3-dimensional network is shown in globular forms alternated with void spaces due to dried whey (Sah et al., 2016). The DASCPEB microstructure variation was observed by scanning electron microscopy (Figure 5). These microstructure variations showed a characteristic net compared with the control sample, whereas the SCPE fraction that increases the branched network was more separated. A high amount of substituted SCPE (40, 50, and 60%) results in a network with bigger void zones (Figure 5E, F, and G), and in contrast, a lesser amount of substituted SCPE (10, 20, and 30%) did not show a considerable change (Figure 5B, C, and D). This gel-network arrangement pointed out a particular protein aggregation to the pH decrease and microstructure formation. By having a net with more separated voids, there is a loss or escape of the serum contained inside, and therefore, a less firm gel (Lee and Lucey, 2004). These may have a relationship with the apparent viscosity obtained and profile aggregation. The results are in agreement with those of Sah et al. (2016), which describe a remarkable difference in beverage microstructure when it is supplemented with prebiotics, rather than when it is not supplemented.

Figure 4. Aggregate size depending on pH for each treatment. (A) Control (0%), (B) low substitution dairy single-cell protein extract (SCPE) beverages, and (C) high substitution dairy SCPE beverages. Experiments were carried out by mixing SCPE with milk in fermentation flasks in the following proportions: 0, 10, 20, 30, 40, 50, and 60 mL/100 mL (shown here as percentages). Graphics show the aggregation profile and the starting point to increase the size of each sample.

Microcapsule Formation

The emulsions with 1 and 2 g/100 g of surfactant did not show stability in an evaluated period and exhibited a flocculation phenomenon (Supplemental Figure S3, <https://doi.org/10.3168/jds.2019-16729>). On the other hand, the 3, 4, and 5 g/100 g of surfactant emulsions showed stability during the 45 min that they were assessed. This stability was related to drop formation (Supplemental Figure S4, <https://doi.org/10.3168/jds.2019-16729>) given by the surfactant capacity to reduce the interfacial tension and encapsulate the OA. The drop distribution has a Gaussian distribution, and $D[4,3]$ was 1.24, 1.58, and 1.71 μm for stable emulsions, respectively. These values increased according to surfactant concentration.

The 4 g/100 g surfactant emulsion was selected for the microencapsulation process and was used for the following experiments. The emulsion was dried, and the microcapsules obtained in scanning electron microscopy exhibited some typical deformations, appearing as softly dented and wrinkled spheres (Figure 7B). This morphology is characteristic of polymeric systems dried by spray drying (Di Battista et al., 2015; Abedi et al., 2016). Particle microencapsulation size and efficiency are the most important quality parameters in microcapsules (Di Battista et al., 2015). Microcapsules

presented a polydisperse distribution (Figure 7A) and the particle size ($D[4,3]$) was 5.18 μm (Table 1). In several previous studies, the microcapsule particle sizes for beverage supplementation were between 1 to 42 μm for oily bioactive compounds and from 2 to 74 μm for others bioactive compounds (Estrada et al., 2011; Toniazzi et al., 2014; Abedi et al., 2016; Comunian et al., 2017; Tan et al., 2018). Therefore, the microcapsules obtained could be used for DASCPEB supplementation. However, MEE was 50.79% and it was low in comparison with what was reported, which was from 75.50 to 90.10% by Estrada et al. (2011) and Abedi et al. (2016) for oily compounds with the same microencapsulation technique. The MEE in the present study might be further enhanced by improving the solids concentration or using other wall materials.

Effect of DASCPEB Microcapsule Fortification

pH of DASCPEB in Storage. Only 30% of DASCPEB was used in the fortification experiment. Supplemented DASCPEB and the control sample showed a pH decrease in the first storage day and a continuous trend for 21 d (Table 2). This might be caused mainly by lactic acid produced from bacteria growth, but with a lower metabolism rate, which is called post-acidification (Comunian et al., 2017). Supplemented DASCPEB

Figure 5. Micrographics for each sample at 5,000 $\times$ in the 6 h of fermentation. (A) Control (0%), (B) 10%, (C) 20%, (D) 30%, (E) 40%, (F) 50%, and (G) 60%. Experiments were carried out by mixing single-cell protein extract (SCPE) with milk in fermentation flasks in the following proportions: 0, 10, 20, 30, 40, 50, and 60 mL/100 mL (shown here as percentages). Micrographics exhibit how as SCPE increases, the microstructure voids increase as well.

S-A had no significant effect on DASCPEB pH regarding the control sample. This result is consistent with those reported by Estrada et al. (2011) and Abedi et al. (2016), which conclude that there was no significant pH difference between the control and fortified beverage samples.

Effect of Microcapsule Fortification in DASCPEB Viscosity. At the time of the microcapsule incorporation, DASCPEB exhibited an apparent viscosity reduction in the control, as a result of mixing operation and microcapsule interaction. The values of apparent viscosity of the supplemented DASCPEB and control samples gradually decreased during the assessed period (Figure 6; Table 2), being more severe in the S-B and S-C samples (3 and 4.5 g/100 g, respectively). As is well known, the storage time influenced the syneresis and the structural rearrangement in the beverage. Several studies confirmed that the microcapsules led to the formation of a weak gel and, as a result, a decrease in viscosity (Comunian et al., 2017; Ghorbanzade et al., 2017; Tan et al., 2018).

Nonetheless, the sample S-A (1.5 g/100 g) did not show a significant difference in the control and had a higher value of apparent viscosity (0.43 ± 0.17 Pa·s) at 21 d. These values are lower than those reported by Comunian et al. (2017) for beverages containing encapsulated echium oil, phytosterol, and sinapic acid at 21 d (0.758 ± 0.08 Pa·s). Several studies reported an increase or very close viscosity values compared with the control after microcapsule fortification in the beverage, though, using other wall materials and microencapsulation techniques (Goyal et al., 2016; Comunian

et al., 2017; Ghorbanzade et al., 2017; Tan et al., 2018). Therefore, it is necessary to carry out more studies to increase viscosity in fortified DASCPEB.

A High Fortification Content Affected the Aggregate Stability. To understand the effect of viscosity on the aggregate size (average particle size), this variable was assessed in storage. The control sample showed a gently increasing size from d 1 to 8; after that time, no changes were revealed (Table 2). An adverse effect was shown in the particle size as a result of fortification. The S-A and S-B particle size increased at the 8th day, and after that, it decreased. The particle size of S-C sample was reduced during the storage time. The rearrangement mentioned in the previous section is evidenced by aggregate size, being more severe in S-C. The beverage is a gel involving protein aggregates (caseins) that capture water within them. This structure is affected by the amount of protein, fat, or other components and their thermodynamic compatibility (Comunian et al., 2017; Tan et al., 2018). Arabic gum is known to generate an anionic effect and a steric repulsion in the surface drop in emulsions (Chanamai and McClements, 2002). This feature makes the microcapsule wall material to generate a steric repulsion, and therefore, it can interact with the cross-linking proteins, generating a disaggregation.

A Small Number of Microcapsules Incorporated Did Not Show a Significant Difference in the DASCPEB Microstructure. The different treatments and control samples did not show significant differences on d 1 (Figure 7), other than some network voids occupied by the microcapsules (Figure 7G and

7I). On d 21, the control and S-A samples exhibited a normal network with regular void (Figure 7D and 7F), so it is evident that the gel microstructure was not affected by the 1.5 g/100 g microcapsule incorporation. By contrast, significant changes occurred with S-B and S-C samples, which displayed a strange change in the network (Figure 7H and 7J), which collapsed, and cracked surfaces replaced it. These observations can be explained by thermodynamic incompatibility between the polysaccharides existing in the wall microcapsule and the caseins (Sah et al., 2016). Nonetheless, it was necessary to assess these interactions and their thermodynamic behavior through MD simulations.

κ -Casein and Molecular Interaction

The predicted κ -casein structure by I-Tasser had a C-score of -4.13 . This value must be between -5.2 and 2 for a good structure prediction (Zhang, 2008). The MD simulations showed that the κ -casein solvated in water had an average bond energy of 928.5 kcal/mol and the κ -casein-glutathione was 910.1 kcal/mol. This loss in bond energy was due to the κ -casein-glutathione interaction (Supplemental Video S1, <https://doi.org/10.3168/jds.2019-16729>). The glutamate from glutathione formed an ionic bond with the amino group from 139

κ -casein glutamate and the glycine from glutathione formed a hydrogen bond with the 87 κ -casein alanine (Supplemental Figure S5, <https://doi.org/10.3168/jds.2019-16729>). The interaction was maintained until the end of the simulation and here we can conclude that SCPE-microcapsule components can interact with milk proteins and help to form a structure in DASCPEB microstructure.

On the other hand, the κ -casein solvated with glutathione, Arabic gum, maltodextrin, and OA exhibited a bond energy average of $1,157.9$ kcal/mol. This energy increase was due first to a molecule increase in the simulation box, and second because there is a formation of 2 clusters: one formed by the Arabic gum interacting with κ -casein and the other one by the interaction of glutathione, Arabic gum, maltodextrin, and OA until the simulation end (Supplemental Video S2, <https://doi.org/10.3168/jds.2019-16729>). The structure formed by the interaction of SCPE-microcapsule-milk proteins was affected for a greater affinity toward Arabic gum. This fact could explain the observations shown in experimental assays, referring to microstructure affectation due to microcapsule incorporation.

As mentioned above, *C. utilis* has endogenous metabolites and cell-wall components, which might contribute to the improvement of host health allowing their ap-

Figure 6. Apparent viscosity for supplemented dairy single-cell protein extract beverages (DASCPEB) in storage. The graphic shows how the microcapsules and time affect DASCPEB apparent viscosity. Error bars represent SD.

Figure 7. Microcapsule micrographics and supplemented dairy single-cell protein extract beverages (DASCPEB). (A) Microcapsules at 5,000 $\times$, (B) microcapsules at 1,000 $\times$, (C) C30 (control) on d 1, (D) C30 (control) on d 21, (E) S-A on d 1, (F) S-A on d 21, (G) S-B on d 1, (H) S-B on d 21, (I) S-C on d 1, and (J) S-C on d 21. Images A and B show the typical microcapsule morphology. Images C to J display the different treatments and how microcapsules and time affect DASCPEB microstructure.

plication in a large range of functional foods (Buerth et al., 2011a; Tomita et al., 2012). These bioactive compounds, known as postbiotics, have been recently shown to present a wide range of biological activities including antioxidant, anti-hypertensive, immunomodulatory, and anti-inflammatory activities (Tsilingiri and Rescigno, 2013; Aguilar-Toalá et al., 2018; Malashree et al., 2019). The mechanism of postbiotics bioactivities associated with the metabolites and cell-wall components released by *C. utilis* still have to be elucidated through in vitro and in vivo studies.

The MD simulations showed a possible interaction between one component from *C. utilis* extracts, as is the glutathione, and one protein from the milk, as is the κ -casein. However, this interaction was affected

by the microcapsule wall materials incorporated, such as the Arabic gum, which has a greater affinity with κ -casein. The MD simulations can help us understand the interaction of the molecules in a product formulation, turning into a very useful tool for rational design.

Table 1. Feed emulsion and microcapsule characterization ($\pm$ SD)

Sample	Sample dried in pilot spray dryer
Drop size emulsion D[4,3] (μ m)	1.58 $\pm$ 0.06
Total suspended solids	23.3 $\pm$ 0.3
Microcapsule size D[4,3] (μ m)	5.18 $\pm$ 0.62
Total oleic acid (g/g of microcapsules)	0.150
Total free oleic acid (g/g of microcapsules)	0.074
Microencapsulation efficiency (%)	50.80 $\pm$ 0.35

Table 2. Effect of microencapsulated oleic acid in fortified dairy single-cell protein extract beverages ($\pm$ SD)

Beverage type	Storage period (d)			
	1	8	15	21
pH				
C30 (control) ¹	4.25 $\pm$ 0.030	4.10 $\pm$ 0.005	4.03 $\pm$ 0.004	4.01 $\pm$ 0.004
S-A ²	4.26 $\pm$ 0.004	4.10 $\pm$ 0.013	4.08 $\pm$ 0.012	4.06 $\pm$ 0.008
S-B ²	4.27 $\pm$ 0.006	4.16 $\pm$ 0.000	4.17 $\pm$ 0.005	4.13 $\pm$ 0.003
S-C ²	4.31 $\pm$ 0.013	4.20 $\pm$ 0.010	4.19 $\pm$ 0.005	4.13 $\pm$ 0.023
Apparent viscosity (Pa-s)				
C30 (control)	1.027 $\pm$ 0.181	0.882 $\pm$ 0.324	0.892 $\pm$ 0.021	0.789 $\pm$ 0.182
S-A	0.499 $\pm$ 0.105	0.413 $\pm$ 0.352	0.288 $\pm$ 0.019	0.431 $\pm$ 0.167
S-B	0.436 $\pm$ 0.058	0.271 $\pm$ 0.007	0.215 $\pm$ 0.112	0.256 $\pm$ 0.042
S-C	0.371 $\pm$ 0.010	0.178 $\pm$ 0.024	0.102 $\pm$ 0.016	0.171 $\pm$ 0.03
Particle size D[4,3] (μ m)				
C30 (control)	9.91 $\pm$ 0.269	10.60 $\pm$ 0.283	10.65 $\pm$ 0.636	10.50 $\pm$ 0.00
S-A	8.61 $\pm$ 0.502	9.57 $\pm$ 0.587	8.25 $\pm$ 0.304	7.98 $\pm$ 0.11
S-B	7.68 $\pm$ 0.820	8.48 $\pm$ 0.106	7.29 $\pm$ 0.311	7.12 $\pm$ 0.22
S-C	7.10 $\pm$ 0.007	7.09 $\pm$ 0.014	6.51 $\pm$ 0.035	6.37 $\pm$ 0.18

¹pH of the control sample before the storage was 4.48 $\pm$ 0.026.

²pH of the samples before supplementation and storage was 4.47 $\pm$ 0.026.

CONCLUSIONS

Candida utilis extracts were successfully used as a substitute for a milk fraction in fermented milk beverages. A 30% vol/vol of substitution was possible without substantial changes in the microstructure of the beverage, but with a viscosity reduction of 0.66 ± 0.077 Pa-s compared with the control sample. Furthermore, the fermented beverage was successfully fortified with 1.5 g/100 g of microcapsules that contain OA, without showing significant changes in the microstructure. However, microcapsule fortification affected viscosity, as well as the storage conditions. The difference is that even though both decreased viscosity, the decreasing behavior observed under storage conditions was normal compared with the control sample. Therefore, it is appropriate to fortify the beverage using microcapsules containing different wall material. The present work aimed to design a beverage model applying multiscale design. This model works as a platform to further engineering dairy beverages in response to the customer's needs and preferences. Additional engineering will be required to improve our model regarding its sensory and organoleptic properties (e.g., color, aroma, taste, and bioactivity), which might influence customer acceptance.

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