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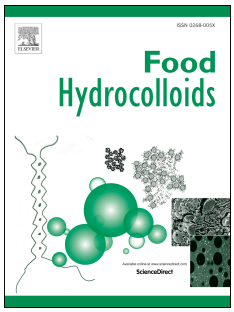

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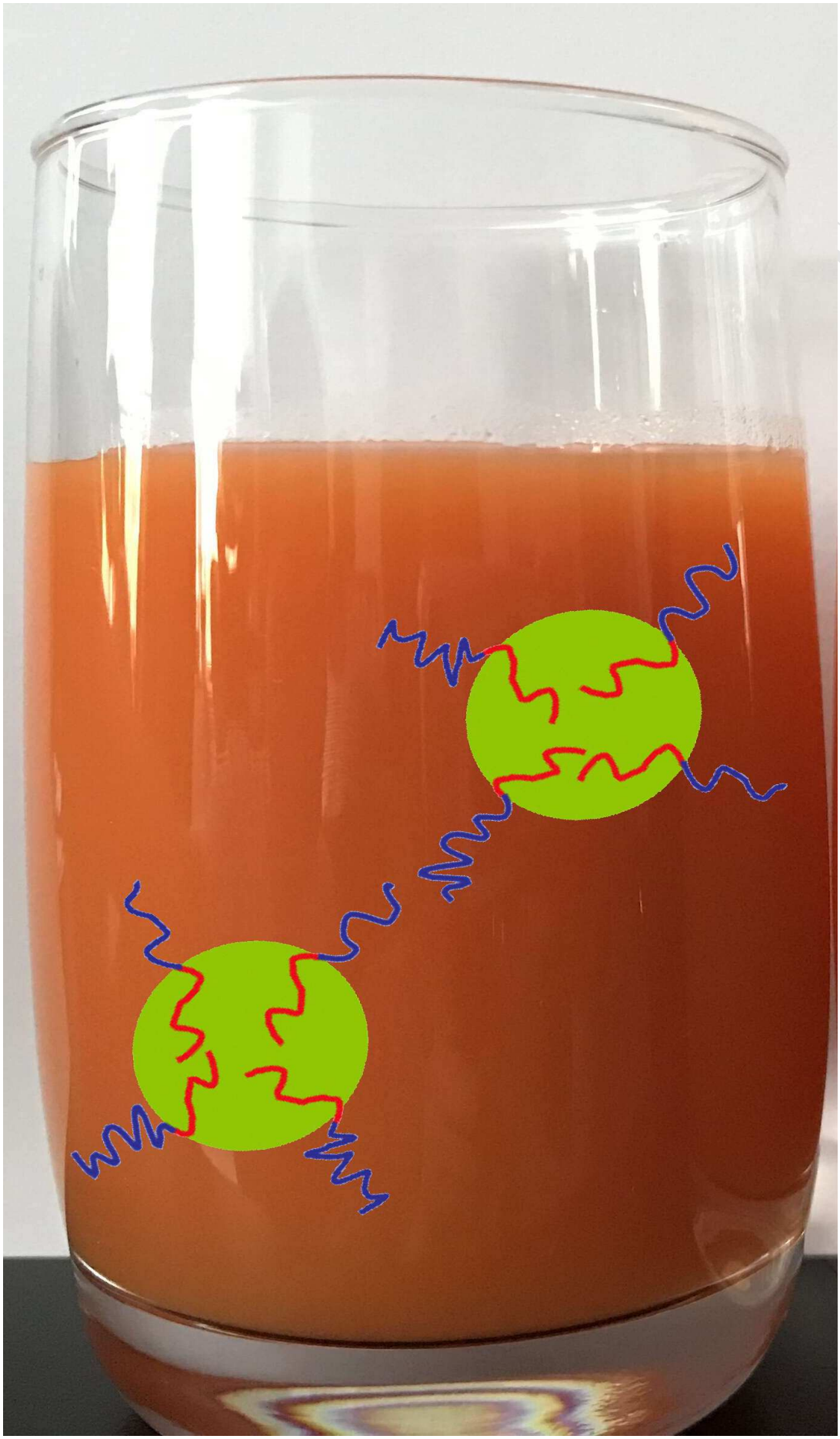

Colloid chemistry approach to understand the storage stability of fermented carrot juice

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Abstract

Probiotic-fermented carrot juice (PFCJ) is a functional food, for which colloidal stability during storage is essential. The water-soluble polysaccharides (WSPs) it contains provide this stability as colloidal stabilizers; they are pectinic, mainly containing galacturonic acid, galactose and arabinose as monosaccharide units. The particle-size distribution, ζ potential, centrifugal sediment ratio, soluble solids content, total acid and structural features of these WSPs were evaluated as functions of storage time. The PFCJ displayed pseudoplastic fluid behavior. Molecular weight and solid morphology of WSPs did not change significantly with storage, although the molecular weights of WSPs showed a slight decrease. The storage stability is ascribed to its WSPs acting as electrosteric stabilizers (which are very robust colloidal stabilizers) by adsorbing onto the insoluble plant cell wall polysaccharides which comprise the dispersed phase in the suspension. Viscosity and particle size remained relatively stable when stored at 4 °C and 25 °C, while centrifugal sediment ratio, soluble solids content and total acid all increased significantly; these can also be explained in terms of the structure of the WSPs. This can provide guidance to choosing ingredients which improve storage stability and other properties of importance to consumers by using the precepts of electrosteric colloidal stabilization: e.g. the presence of water-soluble polysaccharides with long hydrophilic moieties.

Keywords: Carrot juice; Polysaccharide; Colloid chemistry; Stabilization; Physicochemical property

1. Introduction

Fruits and vegetables can be functionally improved by fermentation with probiotics (Sharma, Karki, Thakur, & Attri, 2012), which can add value and greatly improve storage, including during transport (Lee & Salminen, 1995). The carrot known as 'Eastern ginseng' (*Daucus carota* L.) is popular in many countries, and is rich in nutrients. It is commonly processed into juice, but poor colloidal stability of the juice is a significant problem (Xie, Xiong, & Guan, 2014). Our group has developed carrot juice fermented by *Lactobacillus plantarum* NCU 116 (Xiong, Xie, Guan, Song, & Gao, 2013; Zeng, Xiong, Wang, & Huang, 2001), which was found to have better regulating properties for blood glucose, blood lipids, hormones and oxidative stress in type-II diabetic rats than the non-fermented carrot juice. (Li, et al., 2014). It was also found to have improved storage qualities.

This study is about two important properties for storage stability according to colloid chemistry approach: changes in colloidal stability and changes in viscosity of the suspension. For the present purposes, the juice can be considered as a polymer colloid: a water suspension of solid particles comprising water-insoluble cell-wall polysaccharides, mainly cellulose, hemicellulose and water-insoluble pectinic polymers (Galant, Luzio, Widmer, & Cameron, 2014). The characteristic structure of pectins is that they contain (1→4)-α linked D-galacturonic acid (https://pubchem.ncbi.nlm.nih.gov/compound/441476#section=Top); as the largest monosaccharide component of the WSP (Table S1 in Supporting Information) is galacturonic acid, these WSPs are pectinic.

There are two basic mechanisms for stabilizing a colloidal suspension (Goodwin, 2009; Hunter, 2001): electrostatic and steric. Although this subject is well-known in physical chemistry, some of those working in the area of juice stability may not be particularly familiar with it, so a brief summary now follows.

The first mechanism is electrostatic stabilization, whereby charges on the surface of the suspended particles result in the particles repelling each other. These charges are from ionic stabilizers, which can either be chemically bound to the particle, or adsorbed; the latter is when the suspension contains a stabilizer (surfactant) comprising a hydrophobic group and an ionic moiety. The colloidal stability of electrostatic stabilizers is affected by the nature of the charged moiety, and by ionic strength (because high ionic strength can mask the charges). It can also be affected by pH, because the charged moiety may change with pH, e.g. $–O–SO_3^-$ going to $–O–SO_3H$ with a decrease in pH (thereby losing surface charge and hence no longer able to act as an electrostatic stabilizer). The effectiveness of an electrostatic stabilizer is controlled by the inter-particle potential, which can be measured as the ζ potential. It has been observed that changes in ζ potential relate to juice storage stability (Marsanasco, Piotrkowski, Calabro, Alonso, & Chiaramoni, 2015; Schultz, Anthon, Dungan, & Barrett, 2014; Schutz, Barrett, & Dungan, 2014).

Figure 1. Schematic of the mechanism of steric colloidal stabilization.

The second mechanism (which does not seem to have been considered in the literature for stability of carrot juice) is steric, or polymeric, stabilization (Dickinson, 2018; Napper, 1983). Here the stabilizer is a polymer, again with two parts, one hydrophobic and the other hydrophilic. The hydrophobic moiety may be either adsorbed or chemically bonded to the surface of the particle; in the present case, the monosaccharide composition of the WSPs is

such that parts of them will adsorb onto the polysaccharide solids comprising the discrete phase of the colloidal suspension because of hydrogen bonding (which is well known to occur between sugar groups). Colloidal stability arises as follows (this is somewhat of a simplified mechanistic description, but is adequate for the present purposes). If two particles with attached water-soluble polymers (as water-soluble parts of the electrostatic stabilizer in Fig. 1) approach each other, it is entropically unfavorable (high free energy) for these polymers to be compressed together; this tends to keep the particles apart (Figure 1). The level of steric stability is controlled by the chain conformation of the steric stabilizer (if the conformation of the chains is compact in the solvent, they will be less effective as stabilizers (Napper, 1983)), and thus by both the nature of the continuous phase (including changes in ionic strength) and the chemical nature of the water-soluble moiety.

Finally, electrosteric stabilizers are ones which have both ionic and steric properties, for example if carboxylic acid groups on the water-soluble polymer become ionized. These are particularly robust and effective stabilizers, because they have two stabilization mechanisms present in the one species (Dickinson, 2018; Einarson & Berg, 1993; Goodwin, 2009). This is why they are widely used, for example, in latex paints; see, e.g. (Einarson, et al., 1993; Soula, Guyot, Williams, Grade, & Blease, 1999), to give two representative papers on this subject. As electrosteric stabilizers can be charged (depending on pH), the ζ potential will also give information relevant to colloidal stability.

Because of the presence of water-soluble polysaccharides with a range of compositions in fruit juice, it is likely that one or all the above mechanisms may be operative in the storage stability of juices.

If the colloidal suspension (the juice) is colloidally unstable to the extent that there is significant coagulation during storage, this is unappealing to consumers. Consumer appeal and rate of colloidal coagulation are also related to the viscosity of the continuous phase

(water in the present case), as has been observed for juices (Mirondo & Barringer, 2015; J. Q. Wang, et al., 2015; Wojdylo, Teleszko, & Oszmianski, 2014). Changes in viscosity during storage are important for many functional properties of juices: for example, if the viscosity of the suspension increases significantly during storage, the product may be less acceptable to consumers. The viscosity of a suspension is controlled by both the size distribution of the colloidal particles, and by the viscosity of the continuous phase (Dickinson, 2018; Einarson, et al., 1993; Goodwin, 2009). The presence of water-soluble polysaccharides strongly influences the latter, the extent depending on the amount of these and on their molecular weight (and/or molecular size) distribution.

This study aims to understand for the first time the stability of carrot juice fermented by probiotics (mainly composed of *L. plantarum* NUC116) under different storage conditions, using colloid-stabilization precepts. Various physicochemical properties and the basic structure of its constituent polysaccharides were measured. Monosaccharides were measured by high performance anion exchange chromatography (HPAEC), the molecular size distribution was determined by size exclusion chromatography (SEC, a type of gel permeation chromatography, GPC) and the morphology was characterized by scanning electron microscopy (SEM). This is the first systematic mechanistic study of the stability of probiotic-fermented carrot juice.

2. Materials & Methods

2.1 Chemicals and materials

PFCJ was provided by Kuangda Biotech Co. (Nanchang, Jiangxi, China). This was made as follows. Fresh carrots were washed, peeled and then ground into juice. High-fructose corn syrup, which both enhances palatability and increases viscosity (thus increasing colloidal stability, because it slows down the Brownian motion of the particles and thus the likelihood of two colloidal particles having enough kinetic energy to overcome the energy barrier to

coagulation) was added to a final concentration of 8% (v/v). The mixture was then sterilized by pasteurization. After cooling, carrot juice was inoculated with *L. plantarum* NUC116 and fermented at 37 °C for 24 h. Finally, the juice was sterilized at 105 °C for 20 s and packaged.

Monosaccharide standards (L-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, D-mannose, D-fructose, D-glucuronic acid, D-galacturonic acid) and dextran standards for SEC ($\overline{M}_w$ = 5 × 10^4, 8 × 10^4 and 1.5 × 10^5 Da, $\overline{M}_w/\overline{M}_n$ = 1.36, 1.47 and 1.47 respectively, where $\overline{M}_w$ and $\overline{M}_n$ are respectively the weight- and number-average molecular weight) were obtained from Merck Corp. (Darmstadt, Germany) or Sigma Chemical Corp. (St. Louis, USA). All other reagents used were of analytical grade unless otherwise specified.

2.2 Storage stability of PFCJ

Samples of PFCJ were stored at 4 °C, 25 °C and 37 °C. Aliquots of these were collected at 0, 5, 10, 30, 60, 90 and 120 days storage. The particle-size distribution and ζ potential were characterized at 25 °C by a Zetasizer Nano (Malvern Instruments Company, Worcestershire, UK).

The centrifugal sediment ratio, as a measure of colloidal stability of the suspension, was measured by centrifuging at 4070 g for 15 min (the supernatant became clear after 10 min centrifugation). After removing the supernatant, some additional water in the sediment was removed by leaving it spread out on filter paper for 10 min. The sediment ratio was calculated as the ratio of the dried sediment to the total weight of the suspension. The soluble solid content was determined refractometricly (Yan, et al., 2017). The total acid in PFCJ was measured by acid-base titration as described elsewhere (Qiu, Wang, & Gao, 2014). The rheological properties of PFCJ were tested by an ARES-G2 rheometer (TA Instruments Company, New Castle, DE, USA) at shear rates from 0.1 to 1000 s^{-1} at 25.0 °C using a parallel plate geometry (40 mm diameter, 1 mm gap).

158 *2.3 Extraction of the polysaccharide*

159 Polysaccharide was extracted from the PFCJ as follows (M. M. Xu, Yin, Wan, Nie, & Xie,
160 2016). The PFCJ was centrifuged at 4100 g for 20 min, and the supernatant was concentrated
161 in a 70% vacuum at 55 °C with a rotary evaporator; protein was then removed using the
162 Sevag method (Staub, 1965). The resulting modified supernatant was dialyzed against
163 deionized water for 3 days, in dialysis bags with a 3500 Da cut-off at 4 ˚C in excess distilled
164 water which was changed every 8 h. A fourfold volume of 95% ethanol was added to produce
165 precipitation. The precipitate, which contains some ethanol, was redissolved in deionized
166 water. This solution was concentrated under 70% vacuum at 55 °C to remove ethanol and also
167 reduce the volume. The concentrated solution was then lyophilized in a vacuum freeze drier
168 to obtain water-soluble polysaccharide (WSP).

169 *2.4 Polysaccharide composition and characterization*

170 Total sugar content was measured by a phenol sulfuric acid assay using D-glucose as
171 standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The present system contains a
172 number of monosaccharides, and the effect of uronic acids in this case have been pointed out
173 (Guo, et al., 2011). In such cases, glucose is often used to construct the calibration curve and
174 the results are then given as glucose equivalents. Here, both total sugar content and uronic
175 acid content are examined. D-glucuronic acid was used as the standard in total uronic acid
176 content analysis following the sulfate-carbazole method (Blumenkrantz & Asboe-Hansen,
177 1973).

178 The WSP was hydrolyzed by 2 M H_2SO_4 in an oil bath at 100 °C for 4 h. The sample was
179 then diluted with ultra-pure water. The monosaccharide and uronic acid composition of the
180 diluted sample were analyzed by high-performance anion-exchange chromatography (HPAEC)
181 using a Dionex™ ICS-5000 (ThermoFisher Corporation, USA).

182 The SEC characterization was as follows. First, it is noted that particular care is required
183 obtaining molecular weight information for complex branched polymers using SEC

(Gaborieau, Gilbert, Gray-Weale, Hernandez, & Castignolles, 2007). It has not yet been determined whether these WSPs are branched, although this will be the topic of a separate paper; for the present purposes, it is assumed where necessary that they are branched. The size is the SEC separation parameter, the hydrodynamic radius R_h (or the corresponding hydrodynamic volume); see for example (Kostanski, Keller, & Hamielec, 2004). For a complex branched polymer, there is no unique relation between R_h and molecular weight. With differential refractive index detection, as used here, one can obtain the SEC weight distribution $w(\log R_h)$, which is the relative weight, not molecular weight, of polymers with size R_h. It is not recommended to present SEC data in terms of elution time, because that is not reproducible and varies with the SEC set-up and the state of the columns at the time the elugram is obtained (Gidley, et al., 2010). The changes in $w(\log R_h)$ will show whether there was a change in the molecular size distribution during storage. The SEC (Wyatt Technology Co., Santa Barbara, CA, USA) was equipped with a refractive index detector (RI) (Optilab T-rEX, Wyatt, Santa Barbara, CA, USA), an Ohpak SB-G guard column (50 mm × 6.0 mm I.D., 10 μm), SB-806 HQ column (300 mm × 8.0 mm I.D., 13 μm) and SB-804 HQ column (300 mm × 8.0 mm I.D., 10 μm) (Shodex Denko Inc., NY, USA) were used in series. The temperatures of the RI detector and columns were maintained at 35 °C. The mobile phase containing 0.02% (w/w) NaN_3 and 0.1 M $NaNO_3$ was used with a flow rate of 0.60 mL/min. 100 μL sample solution was injected in to the system after passing through a 0.22 μm membrane filter. All data were collected using ASTRA 6.0 software.

The morphology of the WSPs was characterized by scanning electron microscopy (JEOL Ltd, Tokyo, Japan) at room temperature with an acceleration voltage of 5 kV under high vacuum. The procedure was to redissolve 1 mg of sample in 1 mL deionized water, and the solution frozen at -80 °C to make it easier to lyophilize, then lyophilized. The resulting powder was placed in the SEM.

2.5 Statistical analysis

One-way analysis of variance (ANOVA) from Duncan's multiple range test was analyzed by SPSS 22.0 (Chicago, IL, USA), with $P< 0.05$ taken as statistically significant.

3. Results

3.1 Stability of PFCJ during different storage conditions

There were no statistically significant trends in either the particle size or ζ potential of PFCJ with storage time and temperature (Figure 2). Particle size fluctuated in the range 640 - 680 nm. The absolute of ζ potential varied between 19 and 20 mV.

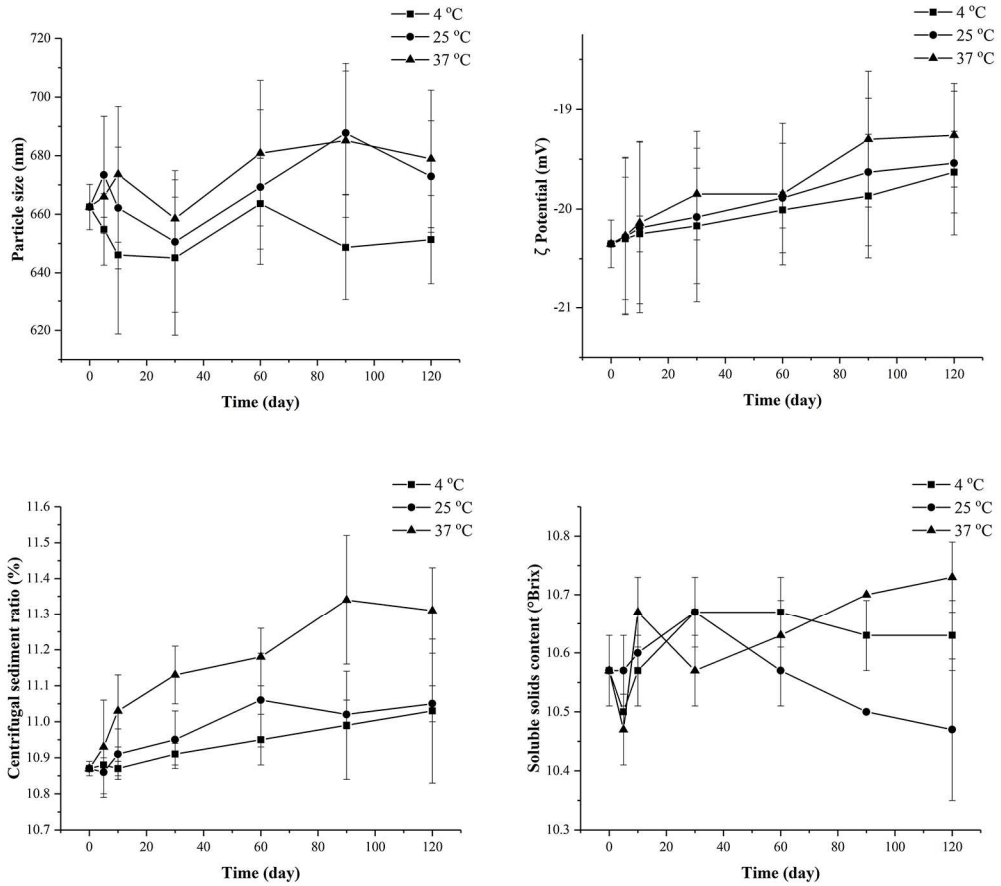
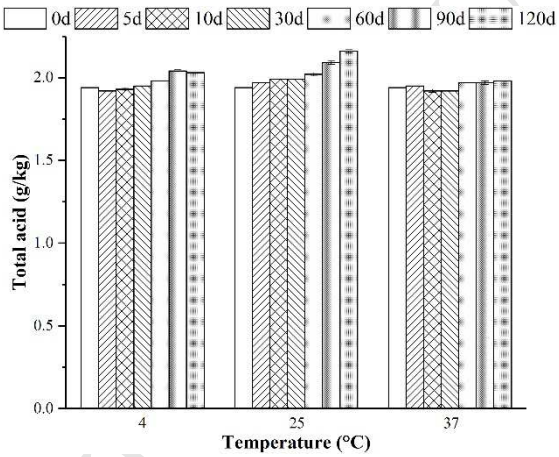


Figure 2. The Changes of physical characteristics of PFCJ under different storage conditions.

219 The centrifugal sediment ratio (Figure 2) of PFCJ increased slightly but significantly with
220 storage time. The rate of centrifugal precipitation increased significantly with storage
221 temperature. There was no significant difference in centrifugal sediment ratio at 4 °C, while
222 significant differences appeared on the 60th day at 25 °C, and somewhat earlier at the storage
223 temperature of 37 °C. The centrifugal sediment ratio of PFCJ fluctuated between around 10%
224 and 11% during the characterization period.

225 The soluble solids content of PFCJ changed slightly but statistically significantly
226 (according to the one-way analysis of variance (ANOVA) from Duncan's multiple range test),
227 from 10.5 to 10.7%, with the increase of storage time.



228

229 **Figure 3.** The total acid of PFCJ under different storage conditions

230 The total acid of PFCJ showed a slight increase with storage time (Figure 3). Significant
231 differences were seen on the 10th day at 4 °C, and on the 5th day at 25 °C and 37 °C.

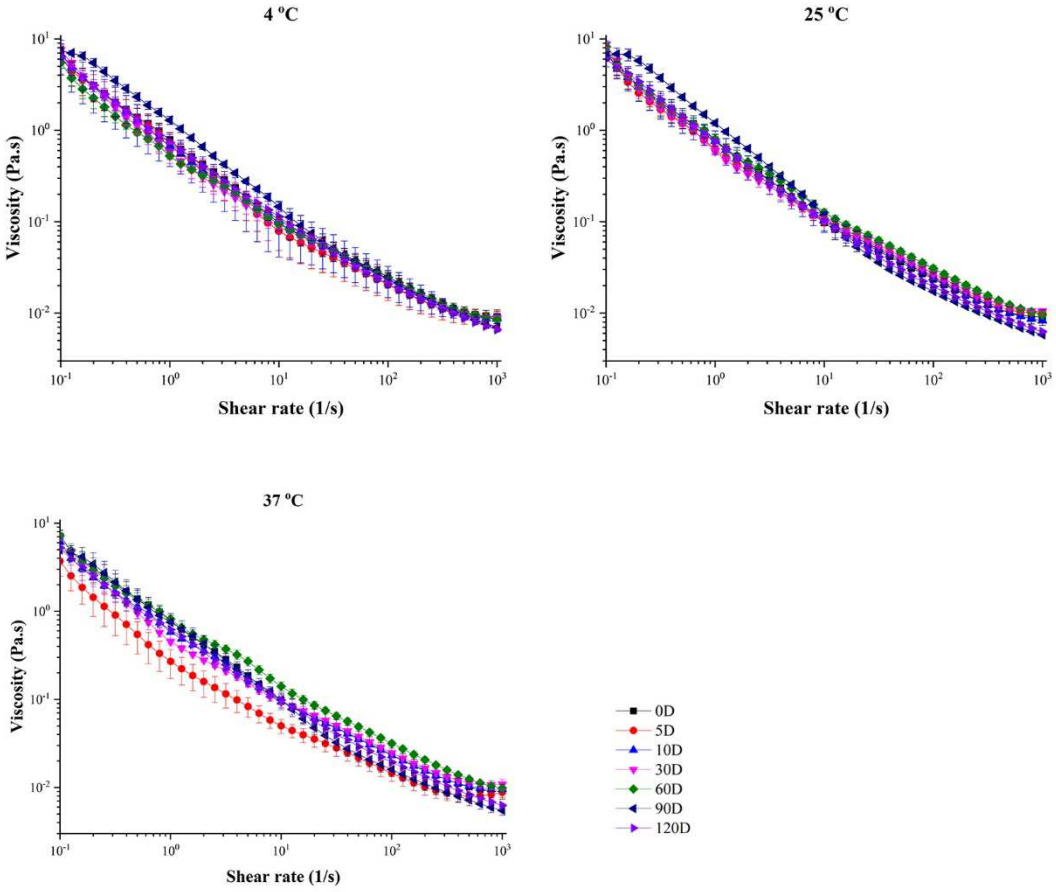


Figure 4. The rheological behavior of PFCJ under different storage conditions.

All the PFCJ under different storage conditions showed pseudoplastic fluid properties, as is typical of a polymer colloid, e.g. (Berend & Richtering, 1995), the viscosity of PFCJ decreasing with increasing of shear rate (Figure 4). The viscosity exhibited similar rheological properties under the various storage conditions, but there were moderate low-shear viscosity changes with time and temperature.

3.2 *Stability of WSP under various storage conditions*

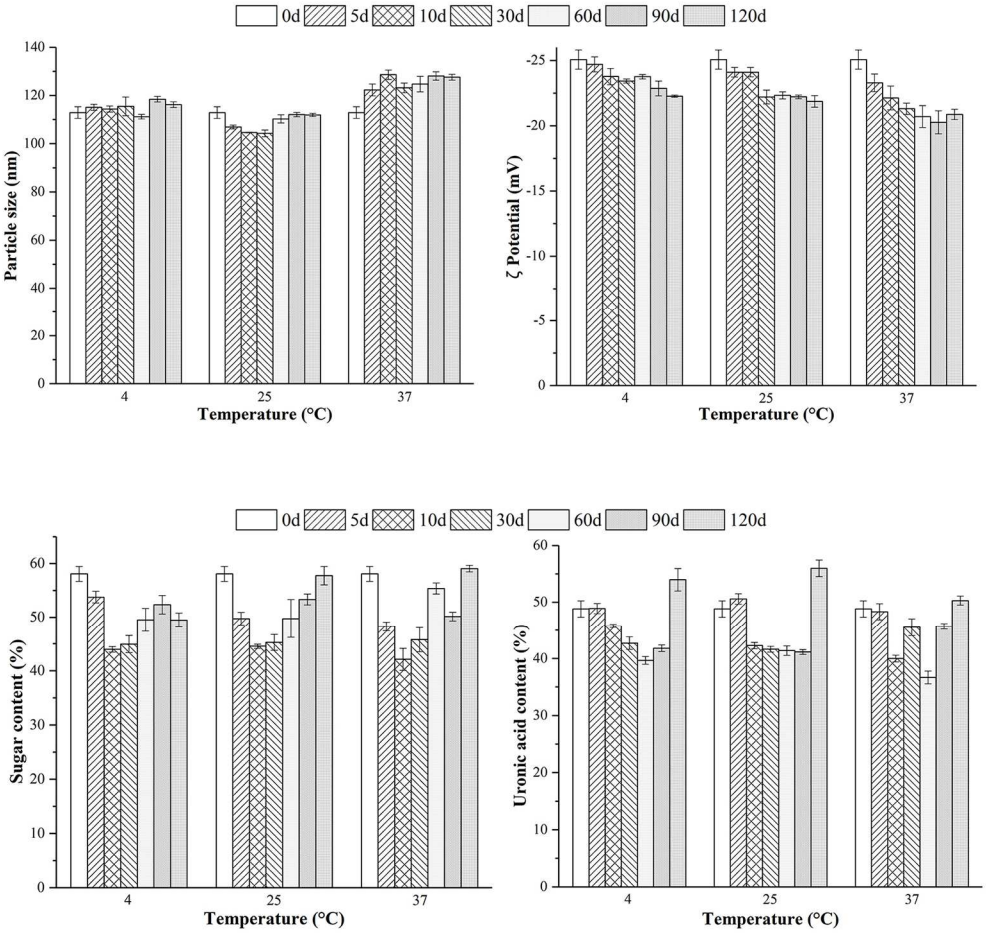


Figure 5. The physicochemical properties of WSP under different storage conditions.

Figure 5 shows the physicochemical characteristics of WSP under different storage conditions. The particle sizes of polysaccharide extracted under different storage conditions were slightly different. The particle sizes were effectively constant at ~110 nm when stored at 4 °C and 25 °C, but varied between 110 and 130 nm when stored at 37 °C. The maximum value was obtained on the 10th day. The trend in the ζ potential of WSP was similar to that of PFCJ, and their absolute values decreased with increasing storage. The most obvious change also happened at 37 °C for WSP. The proportion of sugar content ranged from 40% to 60% under storage, and that of uronic acid varied between 35% and 56%.

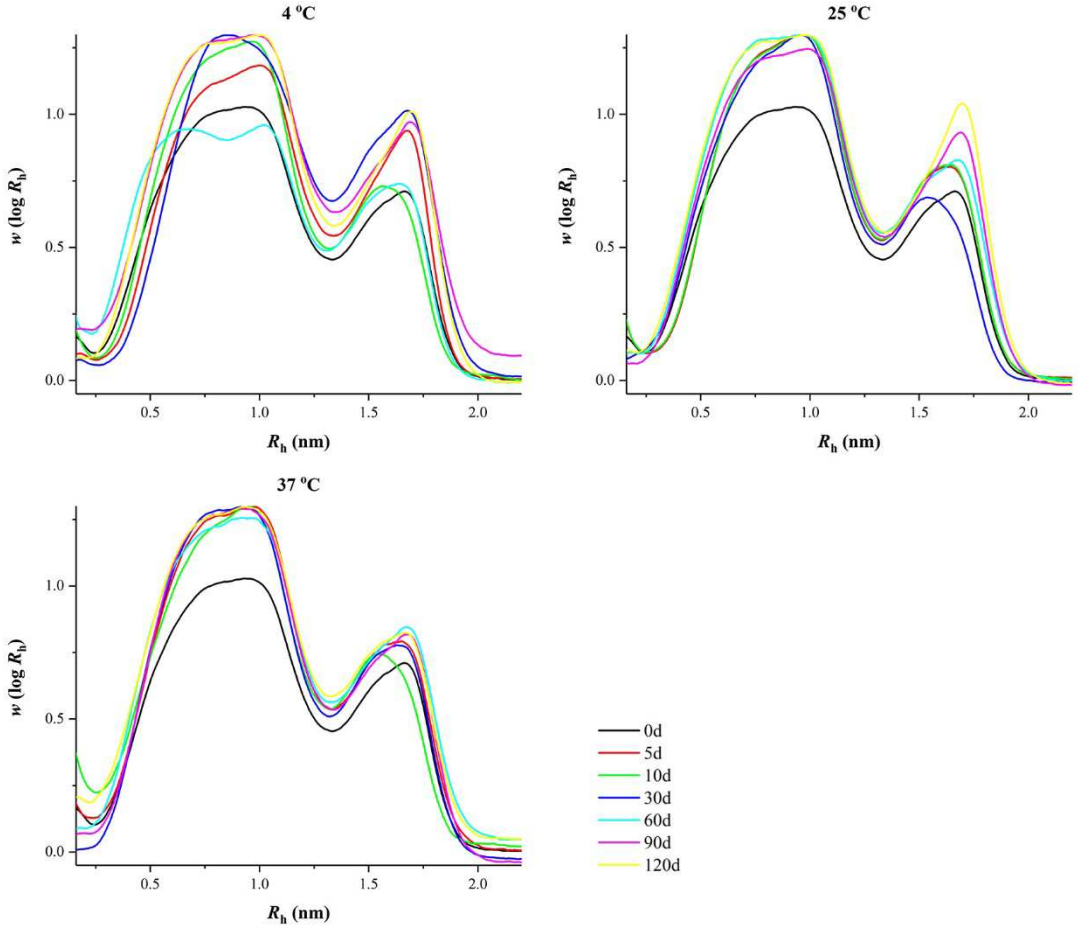


Figure 6. SEC weight distribution of WSPs from fermented carrot juice as a function of hydrodynamic radius of the polymer at different storage times and temperatures.

The SEC results (Figure 6) show that the $w(\log R_h)$ were either bi- or trimodal, and with increased storage time, all components were either constant or moved to slightly lower molecular sizes. The multimodality suggests several different WSP components, as is often seen in natural products such as these (Shi, et al., 2017); compositional analysis of these separate components would be interesting but not relevant to the direct aim of the present paper.

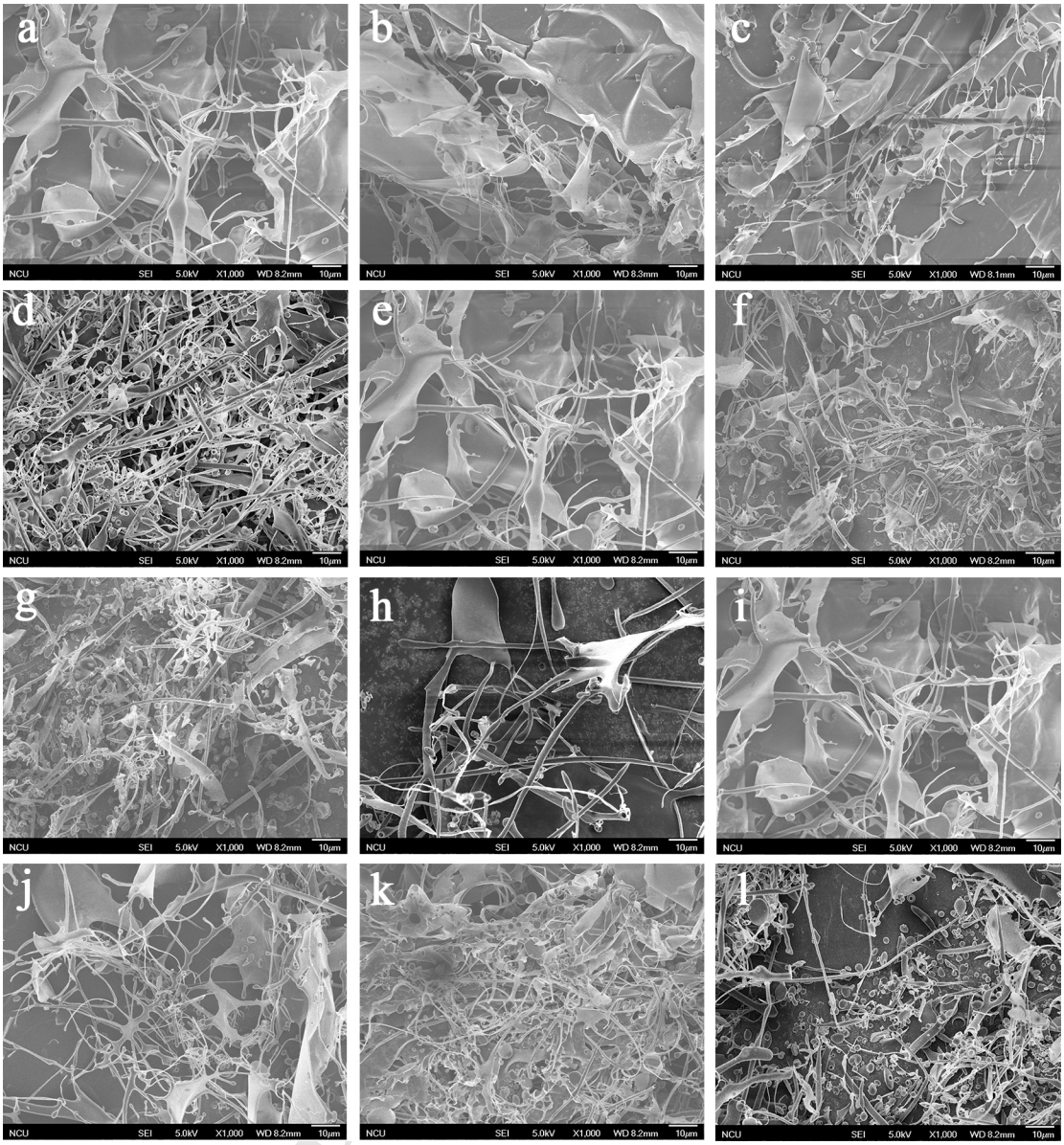


Figure 7. SEM images of WSP under different storage conditions. Letters a-d give solid morphologies of different samples under various storing time periods (0, 10, 60, 120 days respectively) at 4 °C, while letters e-h and i-l give the solid morphologies at 25 °C and 37 °C during the same time period.

SEM shows the presence of both flakes and filaments (Figure 7) in the solid WSP, and there was no obvious difference for WSP obtained from different storage conditions.

4. Discussion

Flavor and taste in the juice are related to its physical properties, such as particle size and acidity (Z. Z. Xu, Lin, Wang, & Liao, 2015). The average particle size of PFCJ was about 650 nm, smaller than that other juices (Schutz, et al., 2014; Zhang, et al., 2016). Total acid values of PFCJ were around 2.0 g/kg, and did not change much at various temperatures in the same period, although with a slight increase over time. It is possible that the slight degradation, manifest in the slight decrease in the SEC molecular size distributions, might be due to formation of some intermediates and/or degradation side-products, e.g. disaccharides (Herraiz & Galisteo, 2002; H. Y. Wang, et al., 2006).

The storage stability of juice is related to its particle size distribution, solid content, centrifugal sedimentation, ζ potential and viscosity, all of which are germane to colloidal stability. If all other factors are constant, colloidal stability (as indicated by centrifugal sedimentation) increases with the absolute value of the ζ potential, the viscosity of the continuous phase, and a more extended conformation and/or higher molecular weight of the (electro)steric stabilizer. However, the present PFCJ system is a complex one, in which all these colloidal parameters may change under storage conditions.

The particle size and soluble solids content of PFCJ did not show significant change over the storage time used here (Figure 2). The rates of centrifugal sedimentation showed no significant changes with time except a small but significant increase at the highest temperature (37 ˚C). The ζ potential of PFCJ did not vary much under different storage conditions, consistent with the good colloidal stability (there being little change in sedimentation rate except at the highest temperature). The viscosity of PFCJ remained stable (Figure 4), and its high value prevented the suspension from layering (Abedi, Sani, & Karazhiyan, 2014). The lack or small amount of change with storage in these various

functional properties, which is technically desirable, shows the colloidal stability of the system, as is typical for systems with electrosteric stabilization.

Certain macromolecules such as pectinic polysaccharides will interact with cations in the system (Aadil, et al., 2015; Klavons, Bennett, & Vannier, 1994), which probably provides electrosteric stabilization and thus maintains the colloidal stability of the juice system. This is because the WSPs would partially adsorb onto the solid particles in the juice, these particles being plant cell wall materials themselves comprising water-insoluble polysaccharides. Water-soluble polysaccharides have been obtained from this PFCJ by centrifugation (M. M. Xu, et al., 2016), and include exopolysaccharides which can be extracted from this *Lactobacillus plantarum* (Zhou, et al., 2017). In the present paper, we consider all the polysaccharides in the fermented juice, irrespective of whether they come from endogenous carrot polysaccharides or are exopolysaccharides. As the aim of this project is to understand the colloid chemistry of the storage stability of fermented carrot juice, there is no need to distinguish exo- and endopolysaccharides.

The suspension was subjected to centifugation and the continuous phase was analyzed. While some steric stabilizers would be incompletely separated from the solid phase by this treatment, analysis of the continuous phase would provide useful knowledge about the stabilizer composition. The particle size of WSP was increased at high storage temperature (Figure 5), consistent with the decreased colloidal stability at higher temperatures. The neutral sugar, and uronic acid, and molecular of WSP changed slightly under different storage conditions, but there was negligible change in the WSP monosaccharides of WSP with storage temperatures (Table S1). The monosaccharide composition of the total WSPs is sufficient for understanding the colloidal stability: the component monosaccharides are such that the polysaccharides can adsorb onto the particles in the suspension (NMR could provide linkage information of the structures of the polysaccharides (Izydorczyk & Biliaderis, 1995),

but such information is irrelevant to the aim of the paper). This suggests no major structural change in the WSPs except for some slight molecular weight degradation. While a fixed number of shorter (electro)steric stabilizers would by itself result in decreased colloidal stability, what is happening here is that the slight decrease in molecular weight would be accompanied by an increase in the number concentration of these polymers, which in isolation would increase colloidal stability; the two effects would seem to largely compensate for each other in the present system. A knowledge of the nature of products formed in the slight degradation is not needed for the aims of this paper.

As stated, this is a complex system and therefore it is not a simple matter to separate the different contributions to colloidal stability. A way of doing this in the future would be to study a variety of juice systems which showed a significant range for all of the structural parameters contributing to colloidal stability. If a sufficient structural range and number of substrates can be obtained, then a conventional statistical analysis, including principal component analysis and correlation coefficients, can separate the effects from each parameter, and then this knowledge could be used to choose components (including additives) to give enhanced colloidal stability. The basis for the relatively good colloidal stability for the WSP-stabilized juices which are the subject of the present study is that these function as electrosteric stabilizers; as is well known in synthetic polymer colloids (e.g. in latex paints) (Gilbert, 1995), these are very robust stabilizers.

In the future, improved storage stability can be gained by using the precepts of electrosteric colloidal stability: for example, seeing if the ingredients are such that the stabilizing polymers have a sufficient number of long hydrophilic chains (which contain some groups which ionize at the juice pH) attached to a hydrophobic moiety.

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346 Province (YC2016-S055).

347

348 # Appendix A.

349 Supplementary data

350

Figure captions

Figure 1. Schematic of the mechanism of steric colloidal stabilization.

Figure 2. The Changes of physical characteristics of PFCJ under different storage conditions.

Figure 3. The total acid of PFCJ under different storage conditions.

Figure 4. The rheological behaviors of PFCJ under different storage conditions.

Figure 5. The physicochemical properties of PFCJ under different storage conditions.

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Highlights:

- Probiotics-fermented carrot juice is a functional food.
- Colloidal stability with storage is essential.
- Changes in molecular structure, viscosity and colloidal properties are examined.
- The good colloidal stability is caused by WSPs acting as steric stabilizers.