Structural and rheological properties of model nutritional beverage emulsions stabilized by bovine lactoferrin: Influence of pH and oil type

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ABSTRACT

The objective of this study was to demonstrate the microstructure and rheological properties of model beverage emulsions containing 1% (w/w) canola oil (CO) or rice bran oil (RBO) stabilized by bovine lactoferrin (bLF) 1% (w/w) subjected to highly acidic and neutral pH conditions. The o/w emulsions were characterized using phase contrast microscopy, confocal scanning laser microscopy and image analysis and rheology. Both CO and RBO emulsions exhibited pseudo-plastic behaviour with the flow behaviour index values being less than 1. Irrespective of pH, a difference between CO and RBO emulsions with respect to median droplet size was observed ($p<0.05$), which might be attributed to the differences in density and viscosity of the respective dispersed phases. The differences in surface hydrophobicity and electrostatic charge of bLF between pH 2 and 7 influenced the emulsion properties. The results might have implications in formulating highly acidic/neutral nutritional beverages containing n-3 and n-6 fatty acids and lactoferrin.

INTRODUCTION

Emulsions and/or emulsion-based ingredients are widely used in food industries to deliver bioactive food components. Such components are proteins, lipids, antimicrobials, antioxidants, vitamins and functional foods, to satisfy the demand of ingredient and formulation innovations[1-3].

Bovine lactoferrin (bLF) is an iron binding globular glycoprotein of molecular weight 78±1 kDa present in milk. It has been a topic of recent research interests partly because bLF has good surface active properties, bioactive functions as well as isoelectric point (pI) > 8[4, 5]. Being surface active, bLF forms electrostatically stabilized emulsion droplets by being adsorbed to the oil-water interface. Furthermore, the high pI of bLF enables bLF-stabilized emulsion to be cationic at neutral pH[6-8]. Besides the emulsifying properties, bLF is a well-characterized biologically active protein with regard to its antimicrobial activity and therapeutic potential[9-11]. The bLF is also known to act as natural antioxidant in food systems containing n-3 polyunsaturated fatty acids (PUFA) and thus increase the shelf life of food products[4, 12, 13]. Based on reviewing relatively limited available literatures on bLF-stabilized systems such as milk and mayonnaise, Jacobsen et al.[14] concluded that bLF shows a concentration dependent antioxidant activity in case of protecting n-3 PUFA against autoxidation[14]. Few earlier reports showed that bLF at 1w/w% concentration was used to stabilize o/w emulsions containing 5-20 w/w% oil concentrations[7, 8, 15]. However, studies involving stabilization of model nutritional beverage system containing lower oil volume fraction and higher bLF concentration was not studied much. The rationale for incorporation of large proportions of bLF in beverage emulsions is not only linked to the potential health benefits. It also gain natural antioxidant properties without adding additional antioxidant, which might be useful in chemical stabilization of PUFA oils in interfacial systems[16].

Lipids are the key nutrients that affect the growth of humans[17]. The balance between n-3 and n-6 PUFAs is essential for human health[18]. The eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the n-3 FAs normally derived from fish, krill or squid oils. The α-linolenic acid (ALA, an essential n-3 FA) and linoleic acid (a n-6 FA) are normally present in many seeds, vegetables and their oils. The impact of n-3 and n-6 FAs derived from fish and plant based oils are detailed in earlier reports[19, 20]. The oils from canola, rice bran, olive, flaxseed, soybean and sunflower seed are the major sources of ALA and are used in conventional foods as baking oils. Normally, the recommended ratio of n-3 to n-6 for human consumption is in the range 1:7 to 1:25[18, 21]. Nonetheless, there have been variations in the n-3/n-6 in the Western diet the last hundred years[20]. For a vegetarian diet the recommended daily intake of ALA is 1.5-3.0 g[22].

The major research challenge of preparing nutritional beverages is related to the potential physical and chemical instability of the emulsions containing CO or RBO. In particular, flocculation, creaming and coalescence influences the physical stability of an emulsion when they are subjected to different environmental conditions [23]. Hence, in the present work, model beverage emulsions containing low concentration (1 w/w%) of CO and RBO stabilized by bLF (1 w/w%) were studied using rheology and microscopy. The emulsions were exposed to pH 2 and pH 7 to investigate the suitability for preparation of both acidic and neutral beverage emulsions, respectively. Extreme pH condition (pH 2) was used to gain insights for designing highly acidic stable emulsions. At present, to our knowledge, there are no collective reports on the microstructure and rheological characterization of o/w emulsions containing n-3 and n-6 fatty acids using bLF as an emulsifier at different pH. The objective of this study was to investigate the influence of oil type and pH on the stability of bLF-stabilized model beverage emulsions containing high protein-oil ratio. The present study is focused on the design of real industrial healthy emulsions using n-3, n-6 fatty acids as well as bLF.

MATERIALS AND METHODS

Preparation of emulsions

The emulsifier stock solution was prepared by dispersing 2.0% (w/w) bLF in 10 mM phosphate buffer at pH 7 containing 0.02% (w/v) sodium azide as an antimicrobial agent, and stirring for 1 hr to ensure complete solubility. The pH of the solution was set to 2.0 (3.0 M HCl) or 7.0 (0.2 M NaOH) as required with continuous stirring for 10 min. The emulsion was prepared by pre-mixing 2 mL 1% (w/w) CO or RBO with 198 mL 1.0% (w/w) bLF solution at 13000 rpm for 1 min (ultra turrax IKA T25) followed by homogenization in four passes in a high pressure homogenizer at 8 kpsi for 2 min (GEA Nitro Soavi S.p.A). The emulsions were stored for 72 hrs at +4 °C before further analysis.

Phase contrast microscopy

A small drop of emulsion was applied over a glass slide with a cover slip, and was viewed at a magnification of 40 x, employing a phase contrast microscope (Model # BX40/F4, Olympus Optical Company, Japan).

Confocal laser scanning microscopy (CLSM) and droplet size distribution analysis

A Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) was used to take the emulsion images. The fat droplets in the emulsions were observed using CLSM. Samples of emulsion (1 mL) were stained with 0.1 mL of 1.0% (w/v) Nile Red. Each stained sample was placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with a cover slip. The samples were then inverted for CLSM analysis after staining. Each sample (spot) was imaged in five places.

Confocal micrographs were analyzed for median droplet size (D50) and cumulative size distribution using image processing software, ImageJ (Wayne Rasband, NIH, USA). The numbers of bins used for droplet size distribution histogram were specified as 100 during size distribution analysis and droplet count. The size distribution analysis was done using the average of three different images taken for one sample. D50 values were used for comparative purpose.

Rheological measurements

A universal controlled stress rheometer (Model #SR5, Rheometric Scientific, NJ, USA) with a coaxial cylinder having an external diameter of rotating bob: 28.8 mm, inner diameter of stationary cup: 30.0 mm was used for the rheological measurements. 30 mL of each sample was used for the experiments. The temperature was controlled by a circulatory water bath and was set to 20 ± 0.1 ^{o}C. Viscosity curves were obtained in the range of shear rate 20-800 s^{-1}. All rheological measurements were carried out in triplicate. Viscosity curves of the oils at 4 ^{o}C were obtained with a Physica MCR 301 rheometer (Paar Physica, Anton Paar, Germany) together with a Titanium CC27 cup and a ST24-2D/2V/2V-30/129 stirrer using same parameters as those of the emulsion.

Statistical analysis

Anova General Linear Model (GLM) in Minitab® 16.2.2 (Minitab Inc., Coventry, Great Britain) was used to compare the size analysis results. For droplet size distribution (Tukey test), all curves (mean of three measurements) were compared. The results presented were based on a dataset of responses from four series containing CO pH 2, CO pH 7, RBO pH 2 and RBO pH 2, respectively. For comparison, the number of counts in the range 0.4 - 9.3 µm were divided into 5 bins (n=10). The sample evaluation type was fixed. The chosen confidence was $p<0.05$ for the variance analysis.

RESULTS

All emulsions were homogeneous, completely milky and pale red during storage at +4 °C. The visual observations showed no phase separation or creaming in any of the emulsion systems during day 16 days of storage.

Table 1. Viscosity and droplet size values of canola oil (CO) and rice bran oil (RBO) emulsions at pH 2 and 7

Oil	pH	$\eta_{emulsion}$ at 500 s^{-1} (cP)	Droplet size of emulsions* (µm)				
			D_{10}	D_{25}	D_{50}	D_{75}	D_{90}
CO	2	1.47	5.2	3.4	2.1	1.1	0.6
($\eta_{dispersed\ phase}$=1.51 cP)	7	1.31	5.5	3.4	1.9	1.0	0.5
RBO	2	1.49	1.3	0.9	0.7	0.5	0.5
($\eta_{dispersed\ phase}$=1.88 cP)	7	1.31	2.0	1.2	0.8	0.6	0.4

* The diameter values (D10, D25, D50, D75 and D90) are based upon percentiles within the distribution of directly measured droplet diameters using image analysis of confocal micrographs.

Table 2. Statistical comparison among different droplet size ranges of canola oil and rice bran oil at pH 2 and 7

	Droplet size (μm)	0.4-2.0 (bin 1)	2.0-3.9 (bin 2)	3.9-5.7 (bin 3)	5.7-7.5 (bin 4)	7.5-9.3 (bin 5)	9.3-18.4 (bin last)
1	CO pH2	20.6^{b}	10.8^{a}	3.6^{a}	1.0^{a}	0.4^{a}	0.1^{a}
	CO pH7	18.8^{b}	5.3^{b}	2.2^{ab}	3.0^{a}	0.6^{ab}	0.0^{a}
	RBO pH2	70.2^{ab}	9.0^{a}	0.5^{b}	1.0^{b}	0^{b}	0^{a}
	RBO pH7	118.2^{a}	4^{b}	0.7^{b}	0^{b}	0^{b}	0^{a}
	p	<0.01	<0.001	<0.001	<0.001	<0.05	>0.05
2	CO pH2	20.3^{b}	15.5^{a}	5.2^{a}	2.4^{a}	1.1^{a}	0.2^{a}
	CO pH7	11.5^{b}	3.4^{b}	2.4^{b}	1.6^{ab}	0.4^{ab}	0.1^{a}
	RBO pH2	1066.7^{a}	8.9^{b}	1.9^{b}	0.3^{b}	0.1^{b}	0.1^{a}
	RBO pH7	63.2^{b}	15.5^{a}	3.1^{ab}	1.0^{ab}	0.5^{ab}	0.0^{a}
	p	<0.001	<0.001	<0.01	<0.01	<0.05	>0.05
3	CO pH2	14.4^{b}	3.6^{a}	2.7^{b}	1.3^{b}	1.0^{a}	0.2^{a}
	CO pH7	30.0^{b}	13.9^{a}	5.9^{a}	2.6^{a}	1.4^{a}	0.2^{a}
	RBO pH2	1294.0^{a}	15.4^{a}	0.9^{c}	0.5^{bc}	0.1^{b}	0.0^{a}
	RBO pH7	145.5^{b}	7.0^{b}	0.7^{c}	0.1^{c}	0.0^{b}	0.0^{a}
	p	<0.01	<0.05	<0.001	<0.001	<0.001	>0.05

^{a,b,c} Mean with different superscripts in a row shows significant differences. The values in rows (with superscripts) indicate the average of counts in different bins.

Microstructure and droplet size distribution

Fig. 1 shows the phase contrast micrographs of emulsions. The micrographs were made at pH 2 and pH 7. The prepared emulsions had similar microstructures and droplet sizes below 10 μm regardless of the pH and oil type. Both CO and RBO emulsions showed a rather uniform distribution of the droplets throughout the volume with appearance of some degree of flocculation.

The confocal micrographs of the CO- and RBO emulsion (Fig. 2) at pH 2 and 7 showed the distribution of droplets similar to phase contrast micrographs. The confocal micrographs analysis after 16 days storage confirmed the emulsion stability during the storage period. The confocal images (16 days storage) are comparable to the phase contrast micrographs (1 and 3 days storage).

The mean droplet size values analyzed using confocal micrograph image analyses are shown in Fig. 2. Table 1 shows the droplet size distributions as D10, D25, D50, D75 and D90 values of emulsions. In the droplet size (μm) versus cumulative size distribution (%) plot, RBO emulsion curve at pH 7 is shifted to the left at pH 2 (Fig. 2). Table 2 shows the results of statistical analysis of droplet size distributions of CO and RBO emulsions. The CO emulsion did not show significant difference in droplet size distributions between pH 2 and 7 ($p>0.05$). The RBO emulsion showed significant differences for the droplet sizes 0.4-3.9 μm between pH 2 and pH 7 (Table 2). Considering CO and RBO emulsions at pH 2 or 7, there were significant differences at respective pH values (0.4-9.3 μm) ($p<0.05$).

Also, this can be observed in Fig. 2. The average droplet size of the RBO emulsions, at both pH 2 and 7, were smaller compared with the CO emulsion droplets at respective pH values ($p<0.05$). More than 40% of the droplets were below 1.0 μm in case of RBO emulsions, whereas only 20% of droplets showed such magnitude of droplet size in case of CO emulsions. A greater number of smaller sized droplets were observed in RBO emulsions (D50=0.7-0.8 µm) as compared to CO emulsions (D50=1.9-2.1 µm) irrespective of the pH used (Table 1).

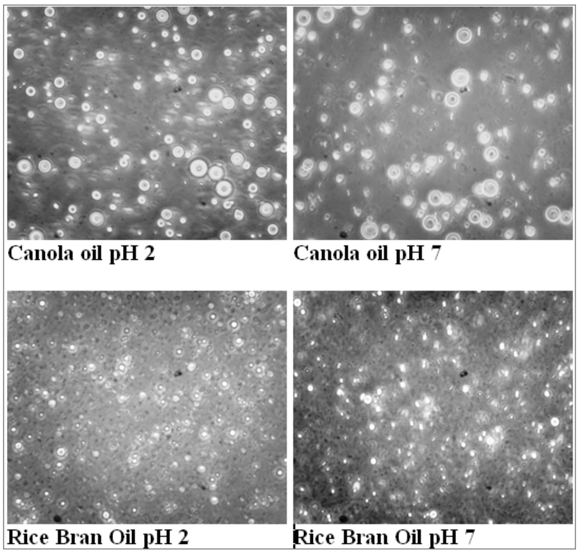

Figure 1: Phase contrast microscopy images of oil in water emulsions in presence of 1 w/w % canola oil, 1 w/w % rice bran oil and 1 w/w % bLF at pH 2 and 7.

<u>Rheological characterization</u>

The plot of viscosity (cP) versus shear rate (s^{-1}) of emulsions is shown in Fig. 3. The emulsions of CO and RBO, at pH 2 and 7 showed shear thinning (pseudo-plastic) characteristics (Flow behavior indices were less than 1). This profile might be attributed to the shear-induced flocculation. At pH 7, both CO and RBO emulsions showed slightly lower viscosity than pH 2. The viscosity values obtained from viscosity curves of CO and RBO are shown in Table 1. Although the viscosities of the dispersed phase (CO and RBO) was significantly different, the profile of all the emulsions showed similar shear-induced pseudoplasticity, which highlights that the rheological behavior was driven by the emulsion structure rather than the oil type.

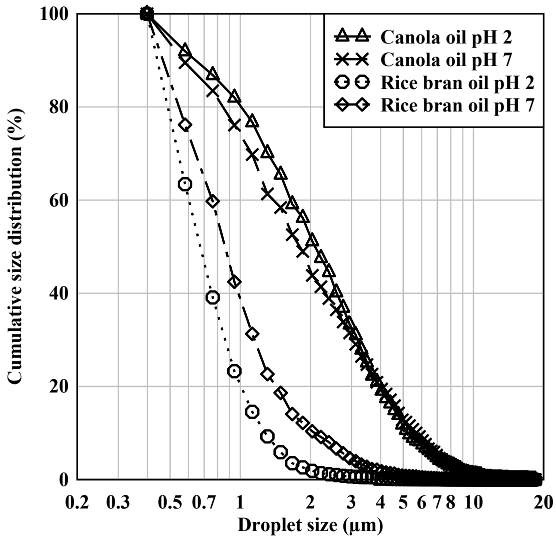

Figure 2: Size distribution plot of oil in water emulsions in presence of 1 w/w % canola oil.

DISCUSSION

The RBO emulsion showed a reduction in droplet size distributions at pH 2 as compared to that of pH 7. This might be due to the emulsion pH being closer to pI (pH~8) of bLF at the oil-water interface.

It is worth highlighting that the protein-to-oil ratio is significantly higher (1:1) in our study as compared to most of the studied literatures (20:1)[6, 8], so depletion flocculation due to the unadsorbed bLF molecules might be expected[24]. However, considering no creaming and aggregation in the systems, it can be inferred that depletion flocculation did not take place.

Shear thinning behavior appeared for all emulsions (Fig. 3), which was probably due to the presence of unadsorbed bLF molecules, which could contribute to the self-association of the bLF molecules and stretching of the unadsorbed globular protein in the direction of the flow. Studies indicate that dilute solution of LF (1-6 mg/ mL) at neutral pH undergoes self-association to form dimers and tetramers in absence of added salts[25]. This concentration is well below the concentration used in our study (10 mg/ mL) and hence some degree of self-association between unadsorbed bLF molecules in pH 7.0 conditions can be expected.). Interestingly, at low pH (pH 2), strong electrostatic repulsion among the positively charged droplets dominated and bLF had a greater surface hydrophobicity at pH 2 than pH 7 as studied previously[26].

The droplet size at both pH values seemed to be dependent on type of oil (CO> RBO). The difference in droplet sizes of CO and RBO emulsions might be attributed to the influence of oil properties such as density and viscosity of CO and RBO on the formation and stability emulsions[27-29]. It is likely that the higher viscosity of RBO as compared to CO (Table 1) might have influenced the production of smaller droplets in RBO emulsions during homogenization irrespective of pH.

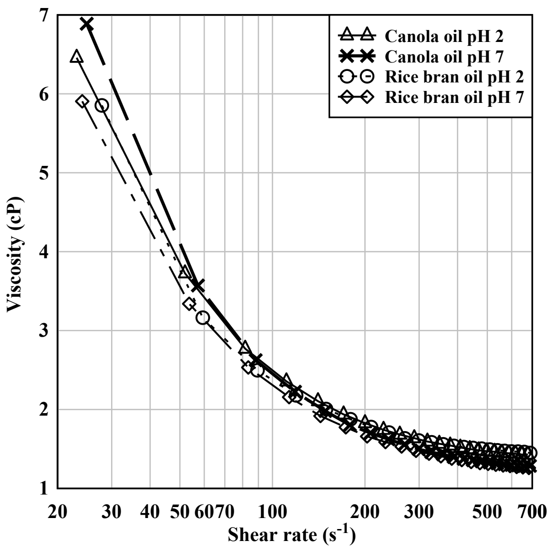

Figure 3: Rheograms of oil in water emulsions containing 1 w/w % canola oil, 1 w/w % rice bran oil and 1 w/w % bLF at pH 2 and 7.

Furthermore, the nature of the oil phase has also been reported to be important with regard to conformation and hence the adsorption of globular protein at the interface[30]. Thus, the emulsion stability seemed to be dependent not only on the pH but also on the type of oil phase.

CONCLUSIONS

The emulsion characteristics reported in the current study might have implications in the industrial formulations of nutritional o/w emulsion based beverages containing low concentrations of oils. The o/w emulsions at pH 2 showed smaller droplet sizes than respective emulsions at pH 7. The RBO emulsions (pH 2 and 7) showed smaller droplet sizes in comparison with corresponding CO emulsions, which might be an indication of the influence of oil type on the physical stability of emulsions. Further stability studies of these emulsions involves longer storage periods and exposure to other environmental factors. Among such factors are ionic strength, addition of sugar and/or other solutes. In addition, heat treatments are needed to enable potential fortification of n-3-rich oils into functional foods and beverages.

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