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Bioavailability of vitamin D encapsulated in casein micelles, compared to its bioavailability in a synthetic emulsifier currently used for supplementation and enrichment

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Abstract

Introduction: Fat-free yogurt was enriched with vitamin D_3 (VD_3) loaded into either re-assembled casein micelles (VD_3-rCMs) or polysorbate-80 (PS80, or Tween-80) micelles (VD_3-PS80). *In-vivo* VD_3 bioavailability was evaluated by a large scale double blind placebo controlled human clinical trial, measuring serum 25(OH)D increases in subjects who consumed fat-free yogurt with 50,000 IU of either VD_3-rCM, VD_3-PS80, or VD_3-free placebo yogurt.
Results:VD_3-rCM and VD_3-PS80 both increased serum 25(OH)D levels by ~8 ng/ml over two weeks, during which no significant differences in mean 25(OH)D levels were observed, evidencing the fact that VD_3 bioavailability in rCM is as high as that in the synthetic emulsifier. Rheological measurements following a shear rate sweep showed that VD_3-rCM yogurt had a higher apparent viscosity than VD_3-PS80 yogurt, and a smaller hysteresis loop. In sensory evaluations, panelists were able to discern between VD_3-rCM and VD_3-PS80 yogurt, and showed a dislike for PS80 yogurt, compared to rCM or unenriched control.
Conclusions: These results complement our past results showing higher protection against thermal treatment, UV irradiation, and deterioration during shelf life, conferred to hydrophobic nutraceuticals by rCM compared to that by the synthetic surfactant or to the unprotected bioactive, in showing the advantageous use of rCM over the synthetic emulsifier as a delivery system for enrichment of food with VD_3 and other hydrophobic nutraceuticals.

תקציר

יוגורט 0% שומן הועשר בויטמין D_3 (VD_3) שעבר אנקפסולציה בתוך מיצלות הקזאין משוחזרות (VD_3-rCMs) או בתוך פוליסורבאט-80 (PS80, או Tween-80) (VD_3-PS80). הזמינות הביולוגית של VD_3 נחקרה על ידי ניסוי קליני מבוקר פלסבו וכפול סמיות, בקנה מידה גדול, בו נבדקה העליה ברמות המטבוליט 25 (OH) D בסרום בנבדקים אשר צרכו יוגורט 0% שומן עם 50,000 יחב"ל של VD_3 שהועשר או ע"י VD_3-rCM או ע"י VD3-PS80 , או יוגורט שלא הועשר כלל ב-VD_3 (פלסבו).
תוצאות: גם VD3-rCM וגם- VD3-PS80 העלו את רמות 25 (OH) D בסרום בכ~ 8ng/ml בשבועיים, במהלכם לא נצפו הבדלים משמעותיים בין הרמות הממוצעות של 25 (OH) D בין שתי הקבוצות שקיבלו VD_3. תוצאות אלה תומכות במסקנה שהזמינות הביולוגית שלVD_3 ב- VD_3-rCM גבוהה כמו זו באמולספייר הסינתטי. מדידות ראולוגיות בקצב גזירה עולה ולאחר מכן יורד, הראו שליוגורט לו הוספו VD_3-rCM היתה צמיגות מדומה גבוהה יותר מאשר ליוגורט -VD_3 PS80, והיסטרזה קטנה יותר. במבחן טעימה, הצליחו הטועמים להבחין בין יוגורט עם VD_3-rCM ויוגורט עם VD3-PS80, והעדיפו משמעותית פחות את היוגורט שהועשר ב-PS80 (גם ביחס ליוגורט הבלתי מועשר).
מסקנות: תוצאות אלו משלימות תוצאות קודמות מקבוצת המחקר שלנו שמציגות הגנה גבוהה מפני טיפול תרמי, הקרנת UV ומפני קלקול הויטמין במהלך חיי מדף, שהוענקו על ידי מיצלות הקזאין לחומרים נוטרסאוטיים הידרופוביים בהשוואה להגנה מועטה ביותר שהוענקה להם על ידי חומרים פעילי שטח סינתטיים או בהשוואה לויטמין בלתי מוגן, מה שמראה בבירור את יתרונות השימוש ב-rCM, בהשוואה לאמולסיפיירים סינתטיים, כמערכת לאנקפסולציה ולהעשרת מזון ב VD_3 או בחומרים נוטרסאוטיים הידרופוביים אחרים.

Introduction

Re-assembled casein micelles (rCMs) closely resemble the structure and function of natural casein micelles: clusters of casein proteins bridged together by calcium phosphate. Hydrophobic nutraceutical (HN)-loaded rCMs are formed by adding ethanolic solution of the HN into caseinate solution while stirring, followed by adding phosphate, citrate, and calcium salts, thereby inducing the self-assembly of the casein molecules into micelles with average size of ~150 nm [1]. Because casein molecules are amphiphilic, the rCM has hydrophobic sites in its core, and can be used as a delivery vehicle for HNs such as fat soluble vitamins, fatty acids, and carotenoids. Specifically, we have previously loaded rCMs with Vitamin D_2 [1], D_3 [2] and docosahexaenoic acid (DHA) [3], nutrients whose addition to food products is highly desirable, but may ordinarily be complicated by their hydrophobicity and susceptibility to environmental stresses. In the rCMs, however, we have found that encapsulated HNs are significantly protected from UV light [1], Thermal treatment [2] and from oxidative damage during shelf life [2, 3]. rCMs have also been loaded with curcumin [4] [5], a natural compound whose efficacy against leukemia cells was reportedly increased when delivered via rCM [5]. In other studied casein micelles were loaded with EGCG [6, 7]. All of these studies substantiate the great potential of using casein micelles as natural vehicles for delivering HNs in functional food products.

Vitamin D_3 (VD_3) is an example of a nutraceutical which is ideally suited for loading into rCMs. There are an estimated one billion people Worldwide who are either deficient in (serum concentrations below 20 ng/ml) or have insufficient (20-30 ng/ml) vitamin D [8], a problem which can be addressed by fortifying foods with VD. After consumption, the liver converts VD_3 to 25(OH)D, or calcidiol, the inactive, but main circulating form of vitamin D. Calcidiol is converted to the active calcitriol [$1,25(OH)_2D$] in the kidneys, from which point it regulates the body's calcium levels [9, 10]. Having sufficient vitamin D also lowers the risk of other diseases, such as cancer [11, 12], type 1 diabetes [13] and cardiovascular disease [14]. However, fortifying foods with VD_3 can be difficult due to its high hydrophobicity [15], and susceptibility to degradation by high temperatures [16] low pH [17], UV[18] and oxidation [19]. A delivery system such an rCM could solubilize VD_3 in an oil-free system and protect it from degradation until the food product is ingested, at which time the casein carrier would break down during gastric digestion and release VD_3 for intestinal absorption.

Previously, we have loaded rCMs with VD_3, used them to fortify 1% milk, and conducted a human clinical trial to study the in-vivo bioavailability of VD_3 in the milk [20], compared to a commercial aqueous supplement based on Tween 80, (a commercial synthetic emulsifier), diluted in water. The results indicated that bioavailability in rCMs was not lower than that in the aqueous VD_3 supplement. In the current study we used VD_3-rCMs to fortify fat-free yogurt, and again conducted a clinical trial to measure bioavailability. Fat-free yogurt is an example of a healthful food product which is the most widely consumed 0% fat milk product. Moreover, due to its lack of fat, it is difficult to fortify with VD_3 using traditional technologies, and without relying on synthetic emulsifiers, thus making it a good candidate for rCM based fortification. For further evaluating the applicability of the technology, we also studied differences in rheology and sensory properties between yogurts fortified with rCMs versus PS80 micelles.

Objectives:

To study the bioavailability of VD when it is delivered in protein nanoparticles (CM) in a fat-free milk product, compared to its delivery within a synthetic emulsifier (polysorbate 80), by an in vivo clinical trial. Additionally, to compare the rheological and sensory properties of yoghurts enriched with the different systems.

Materials and Methods

Materials

Cholecalciferol/Vitamin D_3 (C1357) was obtained from Sigma-Aldrich (Rehovot, Israel). Polysorbate-80 was obtained from Frutarom (Haifa, Israel). Sodium caseinate Caseinate (Casinella QN, lot number 901155) was kindly donated by Kelta Ltd., Israel on behalf of Molkerei Meggle Wasserburg GmbH & CO. KG Germany). Skim milk powder was obtained from Tnuva Ltd., Israel and yogurt culture from Hirshberg Brothers & Co. Chemicals Ltd., Israel, on behalf of Chr. Hansen, Denmark.

Methods

VD_3 loaded re-assembled casein micelles (VD_3-rCM)

To prepare 1L of VD_3-rCM, 10g caseinate powder was dissolved in 500ml deionized water and stirred for at least 4 hours at room temperature. VD_3 powder was dissolved in pure ethanol to give a 13 mg/ml stock solution. 12.5 ml of VD_3 stock solution were gradually poured into the caseinate solution while stirring, followed by the addition of 110 ml of K_2HPO_4 solution (13.9 mg/ml), then 10 ml of tripotassium citrate solution (130 mg/ml), and finally 367.5 ml of $CaCl_2$ solution (4.8 mg/ml) to trigger micelle re-assembly; care was taken to pour the $CaCl_2$ solution slowly, in a thin stream. The VD_3-rCM's final component concentrations were 162.5µg/ml VD_3, 8.8 mM K_2HPO_4, 4.2 mM tripotassium citrate, and 12.0 mM $CaCl_2$.

The VD_3-rCM solution was then homogenized using a Micro DeBee ultra-high pressure homogenizer (Bee Int'l Inc., South Easton, MA, USA). The solution was passed once through an orifice of 0.15 mm, with an average process pressure of 21 kpsi.

VD_3 in Polysorbate 80 micelles (VD_3-PS80)

12.5 mL of VD_3 stock solution (13 mg/ml) were added to 1 L of distilled water containing polysorbate 80, at a 2:1 w/w ratio of polysorbate 80: VD_3. This ratio was based on previous experiments[21] which showed that the particle size decreased with increasing ratio, and reached just below 10 nm at this ratio, while higher ratios did not result in lower sizes. Both VD_3-PS80 and VD_3-rCM solutions were stored at 4°C for up to 24 hours prior to use.

Particle Size Distribution via Dynamic Light Scattering

Immediately following preparation, samples of VD_3-rCM and VD_3-PS80 were diluted 100 fold and their particle size distribution was measured and analyzed via Dynamic Light Scattering (DLS) using a Nicomp™ 380 particle size/zeta potential analyzer (PSS, Santa Barbara CA, USA) at 23°C. The volume weighted particle size distributions were calculated using the Nicomp™ algorithm.

Preparation of VD_3 enriched 0% yogurt

VD_3-enriched yogurt was made by freshly preparing both VD_3-RCM and VD_3-PS80 solutions as described above. The following day, three 12 kg batches of skim milk (0% fat)

were prepared: for 12 kg of milk, 1.32 kg skim milk powder was dissolved in 9.942 kg potable tap water and stirred for 1 hour. Then, 738 g of either VD_3-rCM solution, VD_3-PS80 solution, or distilled water with 12.5% ethanol (for control, un-enriched milk) were added to the milk, bringing the VD_3 concentration to 10 µg/ml. The desired final VD_3 concentration in the finished yogurt was 0.000833% but because the milk was to undergo pasteurization, yogurt fermentation and shelf life, a 20% overage of VD_3 was added. The milk was then pasteurized for 1 minute at 85°C using a plate heat exchanger (APV type JHE, England), and collected in 2 L sterilized plastic jugs. The jugs were wrapped in aluminum foil to minimize light exposure, and stored at 4°C overnight.

The following day, the 2 L jugs of milk were put in warm incubators and periodically shaken until their contents reached approximately 37°C, at which point they were inoculated with 0.17g dried yogurt starter culture for every 1 kg of milk, and shaken gently to disperse the starter. The jugs were then incubated quiescently at 40°C for 4.5 hours, after which they were removed from the incubator, vigorously shaken to break up the curd, and stored at 4°C. One to seven days prior to ingestion, yogurt was poured from the jugs into sterile plastic cups at 150 g portions, wrapped in aluminum foil, and stored at 4°C.

Overall, there were 4 production days, and on each production day all three yogurt types (rCM, PS80, and placebo) were produced.

Yogurt quality control

To verify that the VD_3-enriched milk was successfully pasteurized, and that the yogurt was free of contamination, milk and yogurt samples were collected immediately after processing and sent to Milouda Laboraties, Western Galilee (D.N. Ashrat 25201), Israel. The Pasteurized milk samples underwent a total bacterial count as well as a test for coliform bacteria, and the yogurt product was tested for coliform bacteria, yeasts and molds. In compliance with Israeli Standard 284, all milk samples had total bacterial counts below 50,000 cfu per cc, and coliform counts of <1 per g, and all yogurt samples had coliform, yeast and mold counts of <1 per g, in compliance with Israeli Standard 285.

VD_3 **Quantification from yogurt by RP-HPLC**

Yogurt VD_3 content was evaluated by performing a liquid-liquid extraction based on [2] in a 1 g yogurt sample, and subsequently using reversed-phase HPLC (RP HPLC) to quantify the VD_3 concentration. Twenty µl samples were run on a 4.8x250 mm Vytec™ C-18 column under 1.3 ml/min isocratic flow, in a mobile phase of 80% acetonitrile / 20% methanol. Mobile phase volume was evaluated using integrated peak area, and original samples concentration calculated based on extraction efficiency and dilution factors.

Yogurt VD_3 **content during cold storage**

VD_3-enriched yogurt was stored for no more than 3 weeks, over the course of which samples were taken on at least four different days, and their VD_3 content was extracted and quantified by the technique described above. This was done for yogurt produced on all four of the production days.

Bioavailability evaluation in humans, by a clinical study

An interventional randomized double blind clinical trial was performed, after receiving approval from the local ethical committee [Rambam 0270-12-RMB] and the informed consent of all participating subjects, to assess the bioavailability of VD_3 delivered via rCM-

VD_3 enriched yogurt. 87 subjects (aged 18-61) were selected after completing a physical examination to ensure that they meet the eligibility criteria.

Eligibility Criteria

Potential subjects were excluded based on the following criteria: intestinal malabsorption, lactose intolerance, medical illness (e.g. liver disease, kidney disease, or diabetes), hypercalcemia, excessive alcohol use, pregnancy, use of medications known to interfere with vitamin D metabolism e.g. anticonvulsants, barbiturates, or steroids), granulomatous disease, use of vitamin D supplements, potential for significant sun exposure (e.g., travel to a sunny vacation site or use of tanning beds) within the month prior to, or during, the study.

All subjects who met the eligibility criteria then underwent a laboratory screening of: serum calcium, phosphate, creatinine, albumin and plasma PTH, CBC, ESR, and 25-hydroxyvitamin D [25(OH)D]. This last measurement was used to establish the baseline [25(OH)D] level for each individual.

Within a week after screening, each individual was given a 150g cup of yogurt to consume in its entirety. Subjects were instructed to fast overnight/8 hours prior to ingestion, and 2 more hours post ingestion. Each yogurt cup had been covered in foil and labeled with a unique, randomly generated four digit number. On any given day, the nursing staff was given a group of labeled cups among which there were equal numbers of rCM, PS80 and placebo yogurt. They chose a random cup to give to each individual, and recorded the four digit code. This was to avoid conflating the effects of experimental block (a particular day) and yogurt type. The yogurt consumption date was labeled Day 0, and participants then returned on days 1, 7, and 14 to have their blood samples taken. Serum [25(OH)D] levels were determined by a chemiluminescence immunoassay (CLIA), DiaSorin LIAISON (DiaSorin, Inc., Stillwater, Minnesota), in the Endocrine Laboratory in Rambam Health Care Campus. All samples of each participant were tested on the same kit, to avoid inter-assay variation.

Statistical analysis

Statistical analysis was performed at a significance level of 0.05. Delta 25(OH)D levels were calculated by subtracting each individual's baseline 25(OH)D from all measurements following yogurt consumption. Each data point then underwent a $\log(\Delta 25(OH)D+10)$ transformation; the logarithm was necessary to meet ANOVA requirements and the +10 was to account for negative values. One-way ANOVA was used to compare the three yogurt types, and two-way ANOVA was used to compare the combined effects of day and yogurt type. The Tukey-Kramer adjusted p value was used to account for different samples sizes.

Yogurt rheology

Ten ml yogurt samples were measured using a Brookfield DV2T viscometer (Middleboro, MA, USA), with a bob and cup setup (spindle SC4-21) at a fixed temperature of 10°C. A shear rate sweep was performed from 5 to 120 s^{-1}, and then back down to 5 s^{-1}. Shear stress was measured in Dyn/cm^2, and apparent viscosity (in cP) was calculated as the ratio of the shear stress and the shear rate. Hysteresis was quantified by integrating the area between the up and down shear sweeps on the shear stress/shear rate curve. Samples were measured in duplicate.

Sensory evaluation

Two sensory evaluation tests were performed. The first was a triangle test, to evaluate whether panelists could discriminate between VD_3-rCM and VD_3-PS80 yogurt. Each panelist received 3 teaspoons of yogurt, (two of one type, and one of the other), and was asked to select the sample which was different from the other two. The distribution of yogurt types and order of consumption was randomized. The second test was a preference test, in which each panelist received 3 small cups (~15g) of yogurt, containing VD_3-rCM, VD_3-PS80, and unenriched control yogurt in a random order. The panelists were asked to taste all three yogurts, and assign each a numerical score with 1 being "not tasty at all" and 10 being "tasty". Finally, they were asked to rank the 3 yogurts in order of preference.

Results and Discussion

VD_3-RCM and VD_3-PS80 Particle Size Distribution

The homogenized VD_3-rCMs displayed a bimodal particle size distribution with a majority of micelles distributed about a mean diameter of 89 nm and a smaller population of larger micelles distributed about a mean diameter of 277 nm, while the VD_3-PS80 micelles were all narrowly distributed about a mean diameter of 7 nm (**Figure 1**). This is consistent with our previous findings [20, 21].

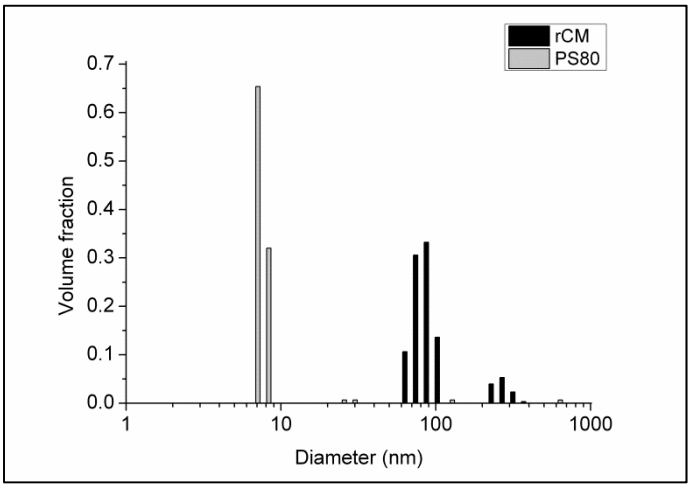

Figure 1: Particle size distribution, VD_3-rCM (black bars), VD_3-PS80 (grey bars).

Yogurt VD_3 content during cold storage

Figure 2 shows the changes in VD_3 content over a three week storage period at 4°C for VD_3-rCM and VD_3-PS80 yogurt. Overall, the differences in vitamin content between the two enrichment methods, and with storage time were insignificant.

Although our experiment was designed to deliver 50,000 IU of VD_3 to each individual in the bioavailability study, it is clear from **Figure 2** that actual yogurt VD_3 content varied slightly by production day, encapsulation method, and storage time. To correct for this variation, we averaged the measured VD_3 concentrations for each production day and yogurt type, yielding eight values ranging from 47,000 to 55,000 IU. Each value was then divided by

51,417 IU, the average of all eight, to yield a dimensionless correction factor. Each participants serum 25(OH)D level was then divided by this factor to account for the relative quantity of VD_3 consumed.

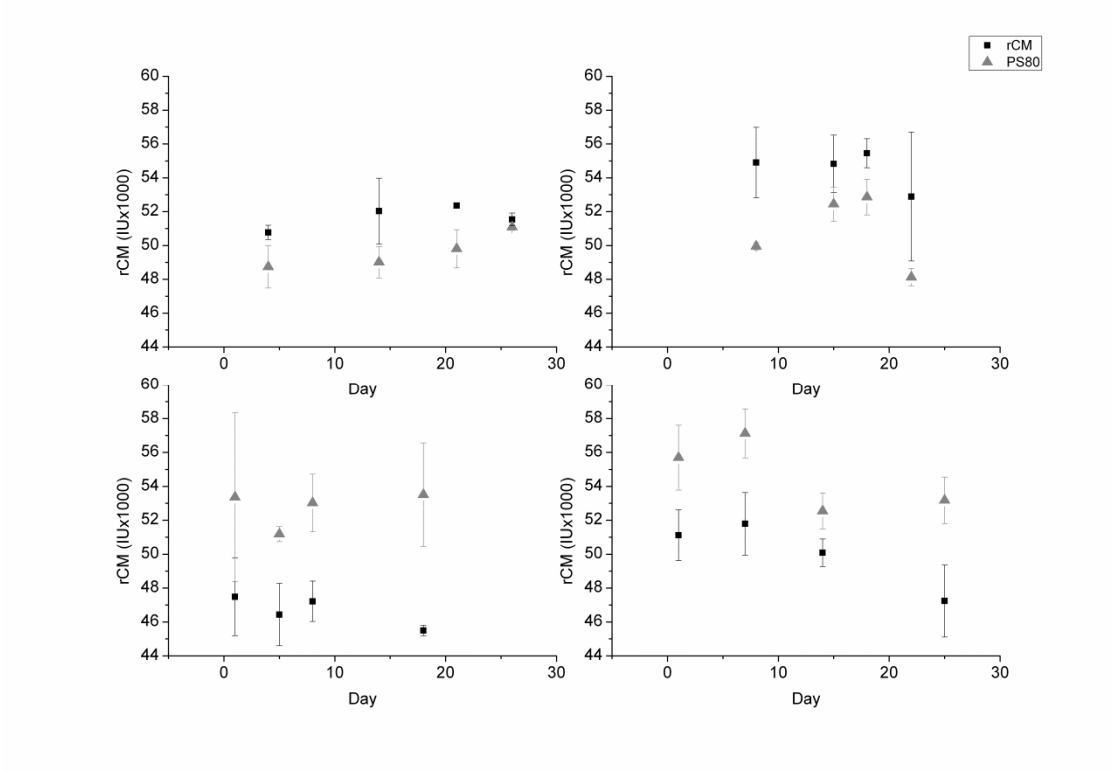

Figure 2: VD_3 content in yogurt during 3 week cold storage. All four production days are shown.

VD_3 Bioavailability

Mean Δ25(OH)D levels were plotted as a function of time (**Figure 3**). As expected, the individuals who consumed placebo yogurt showed no increase in serum 25(OH)D, while those who consumed VD_3-rCM and VD_3-PS80 yogurt showed increases of ~8 ng/ml 25(OH)D after two weeks. There was no significant difference ($p>>0.05$) between mean Δ25(OH)D in individuals who consumed rCM yogurt versus PS80, at any of the times points. Thus, we conclude that VD_3 bioavailability in rCM micelles is not lower than that in the synthetic surfactant.

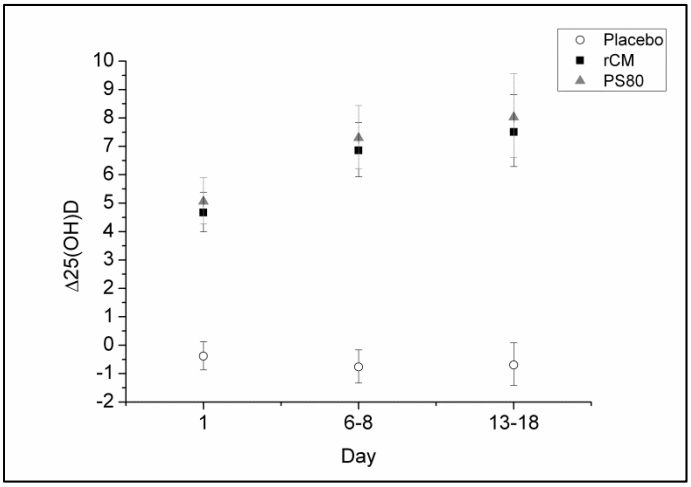

Figure 3: Mean serum Δ25(OH)D following yogurt consumption

Yogurt rheology

All yogurt samples exhibited psuedoplastic, or shear-thinning behavior; as the shear rate increased, apparent viscosity decreased (**Figure 4, center**). The VD_3-rCM yogurt had the highest apparent viscosity over all shear rates, while the VD_3-PS80 yogurt had the lowest. Additionally, the rCM yogurt had the smallest hysteresis loop area, the PS80 yogurt had a larger area, and the unenriched control yogurt had the largest (**Figure 4, right**). The area of a yogurt sample's hysteresis loop is inversely proportional to its ability to rebuild its gel structure after undergoing shear. These results suggest that rCMs slightly improve both the viscosity and gel-rebuilding ability of yogurt. This is most likely due to participation of the rCMs in the formation of the milk casein network. The PS80, on the contrary, decreased the yogurt viscosity, possibly suggesting of disruptive interactions between PS80 and the casein network.

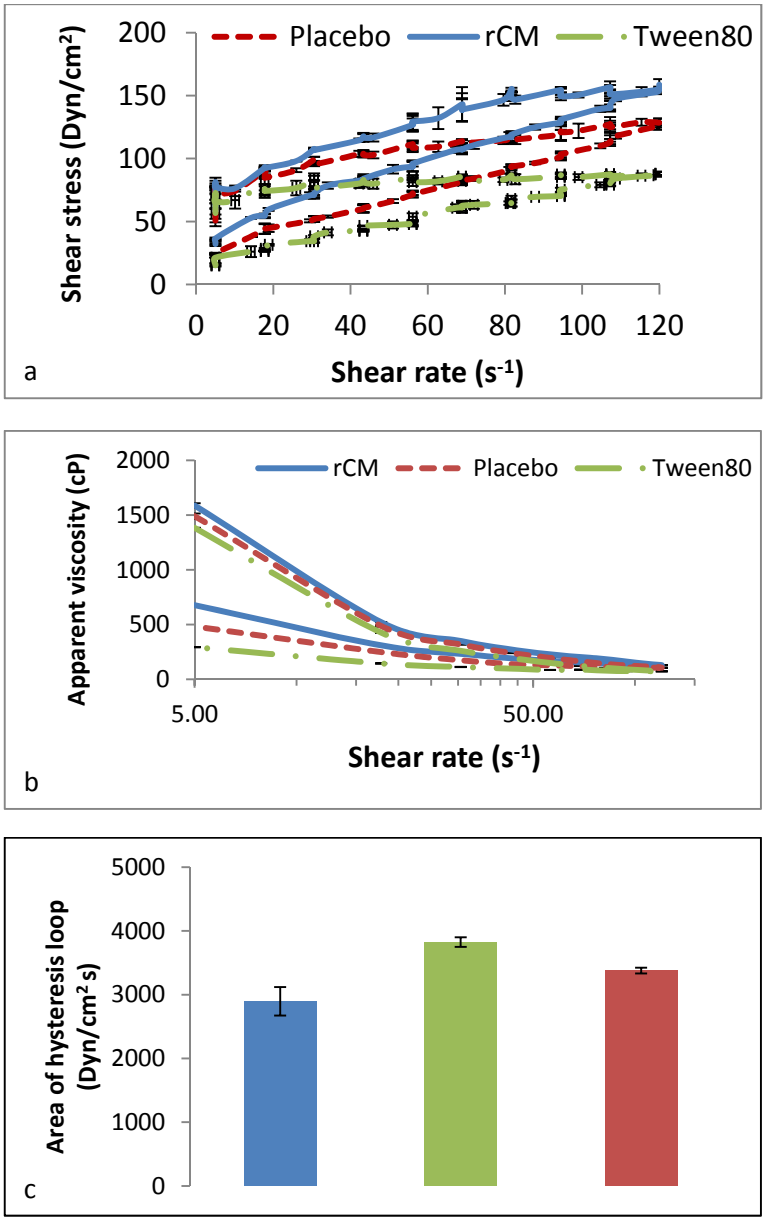

Figure 4: a) Shear stress vs. shear rate b) Apparent viscosity vs. shear rate c) Hysteresis loop area.

Sensory evaluation

In the triangle test, out of 34 panelists, 16 correctly identified the different sample, which meant that the subjects were able to discriminate between the two yogurt types ($p=0.06$). In the preference test, there was no significant difference between the scores of all three yogurt types (**Figure 5**). In order to evaluate the ranking data, the methodology of Newell and MacFarlane [22] was used. We recorded the number of times (x, y, and z, accordingly) each yogurt type was ranked 1, 2 or 3, and then calculated the absolute rank sum as (1*x+2*y+3*z), i.e. the larger the absolute rank sum, the lower the quality-ranking. The results are shown in Table 1. For 34 panelists and 3 yogurt types, a critical rank sum difference of 20 was necessary to establish a significant difference in preference (significance level of $\alpha=0.05$) [22]. Thus, we see that panelists exhibited a significant dislike for VD_3-PS80 yogurt, compared to VD_3-rCM or the unenriched control yogurt.

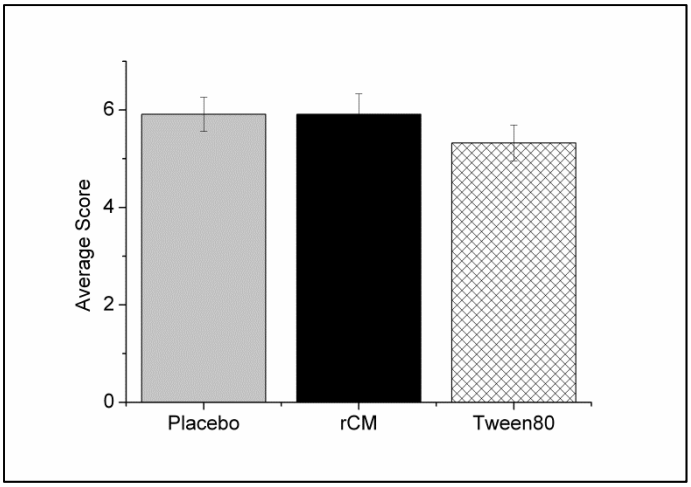

Figure 5: Average yogurt score by type

Table 1: Yogurt rankings by type

	(best)		(worst)	Absolute rank number
	1	**2**	**3**	
rCM	15	12	7	60
PS80	7	6	21	82
Unenriched control	12	16	6	62

Conclusions

In fat-free yogurt, the bioavailability of VD_3 in rCMs was not lower than that in PS80, although PS80 micelles are smaller in diameter. In addition, VD_3-rCM yogurt had a higher overall apparent viscosity, and was preferred in a sensory evaluation, compared to VD_3-PS80 enriched yogurt. These results, complement our previous findings[2] which show that rCM confer better protection to VD3 against degradation during heat treatment, and shelf life, compared to PS80. Moreover, the use of rCM enables an "All-Natural Ingredients" product labeling, while PS80 does not. Taken together these results suggest important advantages of rCMs as a delivery vehicle compared to the industrially used synthetic surfactant.

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