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Antioxidant activity and characterization of whey protein-based beverages: effect of shelf life and gastrointestinal transit on bioactivity

E. ARRANZ^{a, 1}, A. R. CORROCHANO^{a, b, 1}, C. SHANAHAN^c, M. VILLALVA^d, L. JAIME^d, S. SANTOYO^d, M.J. CALLANAN^c, E. MURPHY^a and L. GIBLIN^{a*}

^aTeagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland

^bSchool of Chemistry & Chemical Biology, University College Dublin, Ireland

^cDepartment of Biological Sciences, Cork Institute of Technology (CIT), Cork, Ireland

^dInstitute of Food Science Research (CIAL, CEI UAM+CSIC). Madrid, Spain

¹ E. Arranz and A. Corrochano contributed equally to this work.

*Corresponding author: Linda Giblin

Linda.giblin@teagasc.ie

Abstract

Whey proteins can exhibit antioxidant activity. The objectives of this study were to formulate model whey based beverages with well-established antioxidants (plant polyphenols, vitamins and astaxanthin) to investigate (1) the antioxidant shelf life over a 24-week period and (2) the antioxidant activity after upper gastrointestinal transit. Pilot scale processing (pasteurization, ultra-high temperature or spray drying) was used to prepare beverages which were representative of current product formats. *In vitro* gastrointestinal digestion of test samples was performed using the standardised INFOGEST method and antioxidant activity of samples was determined using ABTS, FRAP and ORAC. Results from the antioxidant shelf life study provided evidence that powder products functionality was preserved. Whey beverages (pasteurized or spray dried) increased or maintained antioxidant activity during gastrointestinal transit. Combination of whey with additional antioxidant ingredients increased the bioactivity of formulated products; however, this greater bioactivity was altered after gastrointestinal transit, depending on processing type and antioxidant methodology.

Industrial relevance

Whey protein-based antioxidant beverages could benefit the elderly consumer to meet their increased protein requirements and boost their antioxidant status. Consumer's acceptance for whey protein-based beverages often improves with clear formulations. This work generated whey protein-based UHT beverages with greater stability and clarity than pasteurised formulations. A novel combination of plant and marine antioxidants increased antioxidant activity of whey protein-based formulations. Furthermore, to suit export markets this work generated spray dried whey protein formulations that did not alter antioxidant potential.

Keywords: whey beverages, heat treatment, antioxidant, shelf life, digestion

Abbreviations:

Whey protein isolate (WPI)

Potassium phosphate buffer (PB)

Alpha-lactalbumin (α -LA)

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Ferric reducing antioxidant power (FRAP)

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)

Oxygen Radical Absorbance Capacity (ORAC)

2,2'-Azobis(2-methylpropionamidine) dihydrochloride (APPH)

Tryptic soy agar (TSA)

Yeast extract peptone dextrose (YPD)

Trolox equivalents (TE)

1. Introduction

Bovine milk contains 3.4% protein, of which, whey accounts for 20%. Whey is composed of the globular proteins; β -lactoglobulin (50-60%), α -lactalbumin (15-25%), bovine serum albumin (6%), immunoglobulins (10%) and lactoferrin (< 3%) (Corrochano, Buckin, Kelly, & Giblin, 2018). The well-documented health-promoting benefits of bovine whey proteins represent an ever-expanding opportunity for beverage formulation. In the last six years, around 4,100 whey protein-based food and beverages were introduced into the USA market (Chavan, Shraddha, Kumar, & Nalawade, 2015).

Bovine whey proteins are rich in branched chain and sulfur-containing amino acids. Several studies have evaluated whey proteins and products as antioxidants and could potentially, be used in beverage formulations to deliver much needed protein and serve to boost antioxidants intake levels of the elderly consumer. Although food pyramids tailored for the over 60s are not currently available, there is consensus among individual countries that diets should be rich in antioxidants and also deliver protein to match the intake requirement of 1g per kg body weight for this life stage (INDI, 2016).

The antioxidant activity of whey proteins is attributed to their hydrophobic and aromatic amino acids which can stabilise electron deficient radicals by donating protons (Mann et al., 2015). Intact whey protein isolate has an antioxidant value of 23,000 μmol Trolox/100 g powder measured by ORAC (Adjonu, Doran, Torley, & Agboola, 2013) and its antioxidant activity is comparable to other protein sources such as egg and soy (Peña-Ramos & Xiong, 2003; Corrochano et al., 2018). Traditionally formulations rich in antioxidants contain carotenoids and/or polyphenols. The marine carotenoid, astaxanthin, extracted from *Haematococcus pluvialis*, has an antioxidant value of 221,000 μmol Trolox/100 g (Régner et al., 2015). Astaxanthin can neutralize free radicals by donating electrons, trapping radicals into its polyene chain or by creating

chemical bonds (Grimming, Kim, Nash, Bickford, & Shytte, 2017). Polyphenols from marjoram extracts exhibit 281,000 $\mu\text{mol Trolox}/100\text{ g powder}$ (Villalva, Jaime, Aguado, Nieto, Reglero, & Santoyo, 2018), whereas individual tea catechins, EGCG (epigallocatechin-3-gallate), 660,830 $\mu\text{mol Trolox}/100\text{ g}$ (Roy, Koide, Rao, Okubo, Ogasawara, & Juneja, 2010). These polyphenols counteract free radicals by H-atom transfer (Papuc, Goran, Predescu, & Nicorescu, 2017). Vitamin B12 is involved in the synthesis of the antioxidant amino acid methionine and its deficiency is associated with cellular oxidative stress (Bito, Misaki, Yabuta, Ishikawa, Kawano, & Watanabe, 2017). Although the antioxidant potential of whey proteins is not as high as plant compounds, they can be incorporated at higher concentrations (22.2% in non-heat treated and 5% in heat treated beverages) into formulations compared to EGCG (less than 0.04%) (Corrochano et al., 2018), astaxanthin (2.26 mg/day) or vitamin B12 (2.4 $\mu\text{g}/\text{day}$). Previously others have combined whey with the antioxidants lutein (Rocha et al., 2017), chlorogenic acid, catechin (He, Yuan, Zeng, Tao, & Chen, 2015) and with *Spirulina platensis* algae extract (Gad, Khadrawy, El-Nekeety, Mohamed, Hassan, & Abdel-Wahhab, 2011). In some cases, whey appeared to have an additive effect on antioxidant beverage activity. These studies did not investigate the influence of thermal processing, shelf life, storage time, spray drying and gastrointestinal transit conditions on the antioxidant activity of these whey combinations.

It is important to note that incorporation of whey proteins into liquid beverages can be technologically challenging. Concentration and pH have been shown to be important factors affecting stability and solubility of whey protein during manufacturing (LaClair & Etzel, 2010). High thermal loads cause whey protein globular structures to unfold exposing hydrophobic and free thiol groups. Interaction of these hydrophobic groups leads to physical aggregation. Free thiol groups will drive covalent aggregation via

sulfhydryl (-SH) and disulfide (S-S) interchange interactions (Considine, Patel, Anema, Singh, & Creamer, 2007; Simpson, 2010). Cornacchia, Forquenot de la Fortelle and Venema, (2014) observed that the extent of aggregation of heated whey proteins in aqueous solutions at 90°C for 30 s gradually decreased if the pH of the solution moved below the pI (whey protein pI = 5.2). At pH 3.5, a solution of 5% whey protein isolate (WPI) heat treated at 90°C for 30 s resulted in a more translucent appearance, a higher dispersibility, a lower reactive –SH group concentration and a more open structure than solutions at pH 4.0 and 4.5 (Cornacchia et al., 2014).

As such, the objectives of this study were to assess the effect of pilot scale thermal processing, spray drying and shelf life on the antioxidant activity of model whey protein-based beverages with additional potent antioxidants (vitamin B12, astaxanthin and marjoram). We also evaluated the effect of the simulated gastrointestinal (SGID) transit on the antioxidant activity of these formulated products to assess their potential to provide an antioxidant boost following ingestion. Moreover, to assess the effect of additional antioxidants on quality parameters of formulated beverages, particle size distribution, viscosity, dispersion analysis, colour, powder moisture, microstructure and microbiological analysis were also determined.

2. Materials and Methods

2.1 Materials

Commercial bovine WPI (Isolac, 91.4% protein content) and α -lactalbumin (93% α -lactalbumin content) were obtained from Carbery Food Ingredients (Ballineen, Co. Cork, Ireland) and Davisco Foods International, Inc. (Le Sueur, MN, USA), respectively. Maltodextrin (with a dextrose equivalent of 16-20) was purchased from Betco Marketing Ltd (Douglas, Co. Cork, Ireland). Astaxanthin (esolvAstraPure 1%

astraxanthin liquid) was acquired from Virun, Inc. (Pomona, CA, USA). Dried marjoram leaves (*Origanum majorana* L.) were obtained from Herboristeria Murciana (Murcia, Spain). Tea polyphenol extract (contained mostly (–)-epigallocatechin-3-gallate (EGCG) (minimum 94%)) was kindly donated by Taiyo GmbH (Schwelm, Germany). Vitamin B12 and all other reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless stated otherwise.

2.2 Marjoram ultrasound assisted extraction (UAE) and HPLC analysis

Marjoram leaves were ground in a knife mill (Grindomix GM 200, Restch, Haan, Germany). Ground marjoram (40 g) was subjected to ultrasound extraction in a ratio 1:10 (sample:solvent) with 70% ethanol. Extraction continued for 30 min using a ½” diameter disruptor horn probe at 70% amplitude (maximum power output of 550 W at 20 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 550; Danbury, CT, USA) at 35°C. After sonication, samples were evaporated, freeze-dried, and stored at -20°C prior analysis.

Phenolic profile analysis of marjoram extract was performed by Agilent HPLC 1260 Infinity series system with a photodiode-array detector (Agilent Technologies Inc., Santa Clara, CA, USA) as previously described by Villalva et al., (2018). The analyses were performed in triplicate.

2.3 Preparation of whey protein-based beverages and powder products at pilot plant scale

2.3.1 Model beverage composition

Two model beverages were formulated at pilot plant scale. The list of ingredients and composition of model beverages are detailed in Table 1. For each of the two experimental repetitions, 40 kg of each formula were prepared. Initially, a base protein

mixture containing 3.52 kg of WPI and 0.08 kg of α -lactalbumin was prepared in 27.50 kg of reverse osmosis H₂O and stored overnight at 4°C under low agitation to allow for complete hydration. The next day, 40 kg batches of each formula were prepared containing 15.55 kg of protein mixture, 3.20 kg of maltodextrin and 21.25 kg of reverse osmosis H₂O for whey beverage formulation (WB). Whey with additional antioxidant ingredients beverage formulation (WBA) was prepared using 15.55 kg of protein mixture, 3.20 kg of maltodextrin, 0.288 mg of vitamin B12, 2.88 g of astaxanthin, 30 g of EGCG, 40 mL of H₂O soluble fraction of 1.2 g of marjoram extract (filtered using 0.45 μ m PVDF filter) and 21.18 kg of reverse osmosis H₂O. Both model beverage formulations were dispersed using a high-shear overhead mixer and pH was adjusted to 3.3 ± 0.1 using food grade phosphoric acid solution 85% (Sigma-Aldrich, W290017).

2.3.2 Heat treatment of whey protein-based model beverages

WB and WBA were heat treated using a MicroThermics Lab heat exchanger (MicroThermics, Raleigh, North Carolina, USA). Initially, pasteurization of each 40 kg batch was performed at a flow rate of 2 L min⁻¹ at 72°C, with a holding time of 30 s. As previously described, incorporation of a preheat step in pilot plant scale UHT plants reduces protein unfolding of dairy beverages (Srichantra, Newstead, McCarthy, & Paterson, 2006). After pasteurization, all samples were cooled to less than 30°C in the cooling section of the heat exchanger. Then 10 kg of each pasteurised beverage was aliquoted into approximately 50 mL volumes and stored at 4°C. Another 15 kg of each of the pasteurised formulas were submitted to ultra-high-temperature (UHT) treatment. Parameters of UHT treatment were a flow rate of 1.3 L min⁻¹ with a preheating of 90.7°C, followed by a final heat treatment of 136°C with a holding time of 4 s. Subsequently UHT samples were subjected to an inline homogenization step (Model

NS2006H, Niro Soavi, Parma, Italy) at 15 MPa. Aseptic filling of samples in 500 mL HDPE sterile bottles was performed and aliquots were stored at room temperature (19-22°C) protected from light. The remaining 15 kg of each pasteurised beverage was spray-dried.

2.3.3 Spray-drying of whey protein-based model beverages

Powders were produced from pasteurised samples of WB and WBA using a single stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark) with a maximum evaporation capacity of 10 L H₂O h⁻¹. A total of 15 kg of each pasteurised formulation was dried with an inlet temperature of 179°C and outlet temperature of 95°C. Spray-dried powder (SD) products were collected and stored in aluminum foil bags at room temperature (19-22°C).

2.4 Characterization of whey-based beverages and powder products

2.4.1 Particle size distribution

The particle size of fresh pasteurised and SD products was measured using static light scattering (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK) equipped with Hydro MV and Aero S feeder units for liquid and powders samples, respectively. H₂O was employed as dispersant of pasteurised samples, absorption index was 0.001 and refractive indices used for the measurements were 1.53 and 1.33 for sample and H₂O respectively.

2.4.2 Powder moisture and microstructure

SD moisture content was determined in fresh samples using a HR83 Halogen Moisture Analyser (Mettler Toledo, Columbus, OH, USA).

Microstructure of SD samples was analysed by scanning electron microscopy (SEM). Each system was attached to double-sided adhesive carbon tabs mounted on scanning electron microscope stubs, and then coated with chromium (K550X, Emitech, Ashford, UK). SEM images were collected using a Zeiss Supra 40P field emission SEM (Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV. Representative micrographs were taken at 1000 \times and 5000 \times magnification.

2.4.3 Viscosity measurement and solid content

The apparent viscosity (at 300 s⁻¹) of the fresh pasteurised and UHT samples was measured using an AR-G2 rheometer (TA instruments, Crawley, West Sussex, UK) equipped with concentric cylinder geometry (diameter 28.02 mm \times length 42.01 mm) at 25°C. Samples were pre-sheared at 300 s⁻¹ for 1 min followed by equilibration step of 1 min. Following this a continuous ramp step was applied to increase shear rate from 1 to 300 s⁻¹ over 2 min, then held at 300 s⁻¹ for 2 min followed by a decreased shear rate from 300 to 1 s⁻¹ over 2 min.

Solids content (% w/w) of fresh pasteurised and UHT formulations was measured using a Smart System 5, Smart Trac (CEM Corporation, NC, USA).

2.4.4 Dispersion analysis

Instability index and sedimentation rates of pasteurised and UHT samples were analysed using an analytical centrifuge LUMiSizer 6112 (L.U.M. GmbH, Berlin, Germany). Samples were initially centrifuged at 576 g for 0.833 h followed by 1,440 g for 3 h, as a proxy to simulate storage periods of approximately 20 days and 6 months, respectively (Murphy, Roos, Hogan, Maher, Flynn, & Fenelon, 2015; Tobin,

Fitzsimons, Kelly, & Fenelon, 2011). Fresh samples without dilution (400 μ L) were placed in polycarbonate cells (2 mm light path). Results were analysed using the software package SEPView 6.0 (Lum GMBH) which calculates instability index and time course separation from the measurement data. For analysis of instability indices of samples, a range of 109-130 mm was used, while for velocity indices a range of 114-121 mm were applied.

2.4.5 Colour determination

Colour was measured in pasteurized, UHT (liquid) and SD (powder) samples using a tristimulus colour analyzer, Chromameter CR400 (Konica Minolta Sensing Europe, Central Milton Keynes, UK). Results were expressed using the Commission Internationale de l'Eclairage defined colour space coordinates L^* , a^* and b^* .

2.4.6 Microbiological analysis

Total counts were determined by filtering sample aliquots (3 or 5 mL) through a sterile 0.45 μ m filter disk. Each disk was aseptically transferred to either Tryptic Soy Agar (TSA) or Yeast extract Peptone Dextrose (YPD) agar. Plates were incubated at 37°C and total numbers were determined by counting visible colonies after 2 (TSA) or 4 (YPD) days.

2.5 Simulated Gastrointestinal Digestion

The static simulated upper gastrointestinal digestion (SGID) protocol developed by Minekus et al. (2014) was followed. Oral phase was not included since samples were liquid formulations. Gastric phase was performed by mixing 5 mL of each beverage with 2 mL of simulated gastric fluids and 2.5 mL of porcine pepsin (EC 3.4.23.1) solution (8,000 U/mL). Then, pH was adjusted to 3.0 with HCl (1 M) and the volume to

10 mL with Milli-Q H₂O. The mixture was maintained at 37°C with continuous shaking for 2 h. The pH was increased to 6.5 to inactivate the pepsin using NaOH (1 M). Intestinal phase was started by adding pancreatin and bile extract (final concentration: 100 U/mL and 10 mM, respectively) followed by a 2-h incubation of at 37°C with continuous shaking. The protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (final concentration: 1 mM) was added to stop the digestion. Samples were then snap frozen in liquid nitrogen and stored at - 80°C.

2.6 Measurement of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The capacity of the beverages to scavenge the synthetic radical ABTS was evaluated following the method described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999) with some modifications. Potassium phosphate buffer (PB, pH 7.0) was prepared by mixing potassium phosphate monobasic and dibasic. Potassium persulphate (5 mM) was used to dissolve 10 mg of ABTS diammonium salt radical (final concentration: 7.3 mM). ABTS stock solution was kept at 4°C in darkness stirring overnight. Immediately prior to use, ABTS was diluted 1:100 in PB and absorbance was checked (0.8 - 1.1) and maintained on ice to preserve stability. Test beverages for self life analysis were diluted 1:3 in reverse osmosis H₂O. Test SD powders were reconstituted in reverse osmosis H₂O to achieve solid content equal to liquid formulations (12.5% WB, 12.56% WBA). SGID and associated undigested samples were diluted 4 times in reverse osmosis H₂O. To start the reaction, 5 µL of test samples or PB (control vehicle) were added to a cuvette which contained 1 mL ABTS and placed in the spectrophotometer.

After 5 min, absorbance was read using a Cary 100 Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 734 nm. Radical inhibition was calculated using the formula:

$$\% \text{ radical inhibition} = [(Abs_{734} \text{ control vehicle} - Abs_{734} \text{ sample}) / Abs_{734} \text{ control vehicle}] \times 100$$

Where Abs_{734} control vehicle is the absorbance of PB with ABTS radical solution after 5 min incubation measured at 734 nm and Abs_{734} sample is the absorbance of the test samples and the radical solution after 5 min reaction. A gastrointestinal control (gut enzymes, bile extract and electrolytes) was included in the experiment and the antioxidant value obtained subtracted from each SGID sample result.

2.7 Ferric Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) of the test samples were assessed following the method developed by Benzie and Strain (1996) with some modifications. A 0.2 M HCl/KCl buffer (pH 2.2) was used in order to avoid protein precipitation. The reagent 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was prepared at 10 mM in 40 mM HCl. The $FeCl_3$ solution (20 mM) was prepared in Milli-Q H_2O . All stock solutions were maintained in darkness and prepared fresh on experiment day. A FRAP working solution was prepared by mixing HCl/KCl buffer, TPTZ-HCl and $FeCl_3$ at a ratio of 10:1:1. This was protected from light and incubated for 1 h at 37°C in a water bath. A stock solution of the synthetic analogue of vitamin E, Trolox, was prepared and serial dilutions (800 - 25 μ M) in Milli-Q H_2O were used to generate a standard curve. To fit in the standard curve, test beverages WB and WBA were diluted 1:2.25 and 1:225, respectively, in reverse osmosis H_2O . SD powders were reconstituted in reverse osmosis H_2O to achieve solid content equal to liquid formulations (12.5%, 12.56%). The

reaction was started by mixing 75 μ L test sample with 1.425 mL of FRAP working solution in black microcentrifuge tubes which were vortexed and incubated for 1 h in darkness at room temperature. Then, tubes were vortexed again and the absorbance was measured at 593 nm on a Cary 100 Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Raw data were corrected to beverage dilutions and results were expressed as μ mol Trolox Equivalents (TE)/100 mL test sample. A gastrointestinal control (gut enzymes, bile extract and electrolytes) was included in the experiment and the absorbance value obtained subtracted from each SGID sample result.

2.8 Oxygen Radical Absorbance Capacity Assay

The ability to inhibit peroxy radicals was assessed by the Oxygen Radical Absorbance Capacity (ORAC) assay developed by Zulueta, Esteve and Frigola (2009a). Beverages were diluted 1:450 in reverse osmosis H₂O. SD Powders were reconstituted in reverse osmosis H₂O to achieve solid content equal to liquid formulations (12.5%, 12.56%) and then diluted accordingly. In black 96 well microtiter plate, 20 μ L diluted test samples, standards or vehicle control (75 mM PB, pH 7.2) were mixed with 120 μ L freshly prepared fluorescein solution (0.117 μ M) in PB,. The plate was incubated for 15 min at 37°C. Meanwhile, 40 mM 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (APPH) radical solution was prepared in preheated PB and kept at 37°C. Then, 60 μ L of APPH solution were added to each well and the fluorescence was measured for 2 h at 90 sec intervals using a Synergy HT BioTek micro plate reader (Winooski, VT, USA). The reaction was completed when fluorescence was less than 5% of the initial fluorescence value. Fluorescence raw data were normalized and the area under the curve calculated. A Trolox standard curve was performed (5 - 80 μ M) and results were expressed as μ mol

TE/100 mL test sample. Data for the SGID control was subtracted from SGID sample results.

2.9 Statistical Analysis

Antioxidant, viscosity, instability index and velocity results were compared using one-way ANOVA followed by Bonferroni's Multiple Comparison post-hoc test using the PASW Statistics 18 software; a p-value less than 0.05 indicated significant difference. Results were expressed as mean \pm standard deviation. Each formulation was produced in pilot plant on two separate runs. Each antioxidant experiment was repeated at least in duplicate and on different days.

3. Results

In total 2 whey beverage formulations, whey protein (WB) and whey protein with additional antioxidant ingredients (WBA) were produced at pilot scale. Both beverages were subjected to mild heat treatment (pasteurization (Pas)), WB-Pas and WBA-Pas. These beverages were then either subjected to an additional UHT thermal load (WB-UHT and WBA-UHT) or spray dried into powder format to suit export market opportunities (WB-SD and WBA-SD).

3.1 Analysis of formulated products

3.1.1 Particle size distribution, powder moisture and microstructure

Figure 1 illustrates the particle size distribution of fresh pasteurised and rehydrated spray dried products. The distribution of WB-Pas was monomodal with a small shoulder of larger size particles (Fig. 1A). Incorporation of the additional antioxidant ingredients in WBA-Pas caused a more homogeneous distribution of particle size. SD samples were

not affected by ingredients composition, since no differences were found in particle size distribution (Fig. 1B). Particle size distribution analysis of UHT samples was not possible due to their clear nature and no minimum obscuration was reached in the Mastersizer.

Moisture % of WB-SD and WBA-SD results are included in Table S1.

Figure 2 illustrates the morphology of WB-SD and WBA-SD. Micrographs show typical shape and structure of spray-dried whey dispersions (Haque, Chen, Aldred, & Adhikari, 2015). The size distribution of the powders were similar in both samples and corroborated particle size distribution results presented in Figure 1. Incorporation of other antioxidant ingredients in WBA-SD (Fig. 3C and D) did not alter morphology of powders compared to WB-SD.

3.1.2 Pasteurized and UHT products viscosity, solid content and dispersion analysis

The apparent viscosity of fresh pasteurised and UHT beverages is shown in Table 2. The viscosity (at 300 s^{-1}) of WB-Pas was $2.47 \pm 0.01\text{ mPa s}$, similar to values obtained with WBA-Pas, $2.54 \pm 0.03\text{ mPa s}$. WB-UHT and WBA-UHT samples resulted in 2.55 ± 0.01 and $2.44 \pm 0.01\text{ mPa s}$, respectively. Therefore, UHT heat treatment did not modify viscosity of samples compared to pasteurization. In addition, incorporation of additional antioxidant ingredients (WBA) had no effect on apparent viscosity.

The solid contents of pasteurised and UHT beverages were measured immediately after processing. WB-Pas and WB-UHT total solid were 11.85 ± 0.04 and $11.68 \pm 0.17\%$, respectively. Similar values were obtained for WBA-Pas, $11.89 \pm 0.08\%$, and for WBA-UHT, $11.76 \pm 0.09\%$.

The physical stability of fresh pasteurised and UHT beverages was measured by means of a LUMiSizer. The transmission of light through the samples is displayed in Figure 3. There were differences in transmission of light through pasteurised (Fig. 3A and C) and UHT (Fig. 3B and D) samples. First profile transmission was 78% for WB-Pas and 55% for WBA-Pas, whereas the last profiles were 85% in both cases. These differences in first profile transmission suggested greater instability of WBA-Pas compared to WB-Pas. In contrast, UHT samples had transmissions close to 90% with no evidence of demixing phenomena. These results were supported by the instability index values (Table 2). Pasteurised beverages had a significantly higher ($p < 0.05$) instability index (0.53 and 0.71, WB-Pas and WBA-Pas, respectively) than UHT (0.04 and 0.11, WB-UHT and WBA-UHT, respectively) after 20 days of storage. The incorporation of additional antioxidant ingredients in WBA did not significantly increase the instability index of pasteurised beverages at 20 days and 6 months of simulating storage. Similarly, UHT beverages instability indices were not affected by the incorporation of additional antioxidant ingredients at studied simulating storage periods. The sedimentation rates (Table 2) were obtained during a simulating storage of 20 days for pasteurised beverages. Results have shown that WBA-Pas had a significantly higher sedimentation ($172.28 \pm 19.88 \text{ mm day}^{-1}$) than WB-Pas ($77.94 \pm 5.54 \text{ mm day}^{-1}$) ($p < 0.05$). On the other hand, UHT values were not reported since visual observations confirmed no sample sedimentation after 6 months of simulated storage.

3.1.3 Colour determination

The colour of the formulated beverages and powders was determined using a tristimulus colour analyser (reflection mode) (Table 3). Incorporation of additional antioxidant ingredients in WB-Pas and UHT beverages significantly increased a^* (redness) and b^*

(yellowness) of the formulations (Table 3, Fig. S1). The increase in red colour is presumably from astaxanthin addition, as esolvAstraPure 1% astaxanthin liquid ingredient was dark red in colour. No differences in lightness (L^*) were found in fresh WBA beverages compared to WB.. The storage of WB-Pas samples increased lightness (higher L^*), and increased yellowness of WBA-Pas samples. No changes were found in WB-SD and WBA-SD with time. . UHT formulations darkened after 24 weeks of storage, with increases in yellow colour.

3.1.4 Microbiological analysis of beverages

The microbiological shelf life of the model beverages was assessed and compared in the case of the pasteurised and UHT treatments. No bacteria or yeast were detected by standard plating methods in the sealed UHT samples during the entire period of the study. Only a single log increase in total counts was observed for the pasteurised samples after 10 weeks (Fig. S2). This microbial stability would be expected given the low pH of the model beverages (pH=3.3) and the 4°C storage temperature. However, the microbial growth could interfere with antioxidant assays so no further analyses of antioxidant activity of pasteurised samples were performed after 4 weeks.

3.2 Shelf life antioxidant activity of formulated beverages

The antioxidant activity of the model beverages under different processing was determined over time by 3 different methodologies (ABTS, FRAP and ORAC) (Fig. 4). WB-UHT exhibited highest ABTS inhibition, FRAP and ORAC values than WB-Pas or rehydrated WB-SD at all time points, with the greatest values at time 0 (38.84% ABTS inhibition; FRAP = 14.55 $\mu\text{mol TE}/100\text{ mL beverage}$; ORAC = 1,481.16 $\mu\text{mol TE}/100\text{ mL beverage}$, $p < 0.05$). However, after 24 week storage, WB-UHT inhibited ABTS by

25.53% (a 13.31% loss), exerted 7.32 $\mu\text{mol TE}/100\text{ mL}$ beverage by FRAP and 833.99 $\mu\text{mol TE}/100\text{ mL}$ by ORAC. Not surprisingly, the inclusion of additional antioxidants (astaxanthin, marjoram extract (composition detailed in Table S2) and vitamin B) substantially increased the antioxidant potential of the 3 formulations, WBA-Pas, WBA-SD and WBA-UHT. Storage time had no significant effect on the ABTS inhibitory activity of the WBA beverages ($p > 0.05$). On the contrary, ORAC and FRAP values of WBA-UHT significantly decreased by 25% after 24 week storage ($p < 0.05$).

3.3 Simulated gastrointestinal transit effect on antioxidant activity

To investigate if these whey-based formulations maintained their level of antioxidant activity during upper gut transit, all test samples were subjected to a static SGID protocol. The harsh conditions of the gastrointestinal tract had no effect in FRAP values of all WB (Fig. 5). However, SGID of WBA samples reduced the antioxidant activity of Pas and UHT and maintain the activity of SD beverages by FRAP. Post SGID, ORAC values increased for all samples to reach 4,755-6,326 $\mu\text{mol TE}/100\text{ mL}$ regardless of formulation or processing type. WBA samples post SGID significantly decreased their ABTS inhibition and reached comparable ABTS values to WB samples post SGID (20%, $p > 0.05$). The antioxidant activity post SGID of WB-SD, WB-UHT, WBA-SD and WBA-UHT samples was preserved after 24 weeks of storage (data not shown).

4. Discussion

Once exposed to the hydrolytic conditions of the upper gut, the antioxidant activity of whey beverages, as measured by ABTS and ORAC, were comparable to whey beverages with additional potent antioxidants. The antioxidant activity of whey beverages (pasteurized or spray dried) was maintained during storage. Interestingly, UHT processing produced beverages of greater clarity and stability compared to

pasteurisation. WB-UHT showed higher antioxidant activity than pasteurized or spray dried samples.

For these beverages to boost antioxidant status of the body, their antioxidant activity presumably should be maintained for as long as possible during gastrointestinal transit. SGID significantly increased ORAC values of all the beverages and maintained (WB and WBA-SD) or reduced (WBA-Pas and UHT) FRAP values. In agreement with ORAC values, Şanlıdere Aloğlu (2012) showed that the antioxidant activity of milk subjected to SGID increases regardless of whether or not it is raw, pasteurized (72–75°C, 15–30 s) or sterilized (121°C, 15 min). Interestingly, ABTS inhibition decreased for all WBA after SGID. Villalva et al. (2018) observed that the antioxidant activity of a marjoram extract significantly decreased from 2.81 to 2.67 mmol TE/g after SGID ($p < 0.05$). The authors also reported that the concentration of phenolic acids present in the extract as rosmarinic acid or luteolin was significantly reduced after SGID while neochlorogenic and *p*-coumaric acids were only detected after digestion. Catechins from tea also underwent a reduction in their FRAP values after being exposed to simulated gut conditions (Record & Lane, 2001). Interestingly, a nanodispersion with whey and astaxanthin survived the gastric digestion but it was destroyed during intestinal transit (Shen, Zhao, Lu, & Guo, 2018). These results may corroborate why the antioxidant activity of all WB samples increased post SGID, but in turn, why WBA samples decreased their ABTS inhibitory activity. Added carotenoid and polyphenols may also interact with the digestibility of whey as observed by He et al. (2015) who found that plant polyphenols, chlorogenic acid or catechin, reduced intestinal whey protein digestibility by 27% compared to the degree of hydrolysis of whey protein alone. This may reduce the release of antioxidant peptides and amino acids and therefore decrease the antioxidant activity of the formulation. There are notable discrepancies between

different antioxidant assays (FRAP and ABTS) with ORAC assays indicating whey beverages performs as well as whey beverages with additional antioxidants for antioxidant activity post upper gastrointestinal transit. Such methodology discrepancies have been observed previously (Adjonu et al., 2013; Corrochano et al., 2018). Whether whey beverages are equal to whey beverages with additional antioxidants after consumption in terms of antioxidant activity can only be resolved by human intervention trials where blood antioxidant biomarkers are measured.

WBA had substantially higher antioxidant capacity compared to WB (pasteurized, UHT or spray dried). However, the combination of intact whey proteins (0.5-6%) with the carotenoid lutein (0.0032%) did not boost the antioxidant capacity of the beverage (Rocha et al., 2017). In addition, intact whey proteins decreased the antioxidant activity of a beverage containing (1:1) algae *Spirulina platensis* although the effect of heat treatment on this activity was not evaluated (Gad et al., 2011). An acidic (pH 3.7) and thermally-treated (121°C, 10 min) polyphenol beverage showed higher ABTS values after addition of 0.2% whey (He et al., 2015).

In our study, UHT heat treatment markedly increased the antioxidant activity of WB samples compared to pasteurisation. Bogahawaththa, Chandrapala and Vasiljevic (2017) described that exposure of whey proteins at high-temperature, short time heating (100°C for 30s) results in native protein conformational changes and usually irreversible denaturation. In contrast, low temperature heat treatment (72°C for 15s) minimizes denaturation of whey proteins. Accessibility of thiol groups may explain the higher antioxidant activity of UHT samples compared to pasteurised samples. However, the results differ from Zulueta, Maurizi, Frigola, Esteve, Coli and Burini (2009b) who showed higher ORAC values for whey from pasteurised milk than whey from UHT milk. This discrepancy may be explained by the use of whole milk by Zulueta et al.

(2009b). Interestingly, the increase of ORAC and ABTS values after heat treatment was masked by the addition of known antioxidants to WB.

Recently, thermal processing using the novel technology Ohmic Heating on a whey-acerola based beverage increased its DPPH inhibitory activity compared to its pasteurized counterpart (8.88 ± 0.31 and 8.48 ± 0.18 $\mu\text{g TE/g}$, respectively) (Cappato et al., 2018). However, the pasteurized beverage showed higher FRAP values (196.27 ± 10.27 $\mu\text{g TE/g}$) than Ohmic Heating sample (248.15 ± 36.28 $\mu\text{g TE/g}$, $p < 0.05$) (Cappato et al., 2018). While the published literature on the effects of technological interventions such as heat treatment and homogenization on antioxidant activity are often contradictory, it is clear that as reported in this study these interventions can have a marked impact on protein structure which can in turn affect antioxidant activity.

A 24 week storage period did not alter the antioxidant activities of WB-SD and WBA-SD samples. However the antioxidant capacities of WB-UHT and WBA-UHT were reduced over the shelf life study. Shah, Mokahe and Mishra (2016) demonstrated that the ABTS inhibition decreased from 55.11% to 40.65% in a whey beverage with inulin (3%), the probiotic culture *Lactobacillus helveticus* (2% v/v), orange juice (10% v/v) and sugar (10%) after 28 days at room temperature. Whey was obtained from filtered double toned milk and boiled at 95°C for 5 min (Shah et al., 2016). Previously, Sady, Jaworska, Grega, Bernas and Domagala (2013) reported that storage for 12 months decreased antioxidant activity of pasteurised orange beverages containing acid whey (0.57% protein content) from 18.5 to 7.9 $\mu\text{mol of TE/mL}$. Similarly to our study, the above authors observed differences in colour analysis of beverages during storage with 6 months storage at 4°C decreasing lightness parameter L^* of their beverages to levels comparable to WB-UHT and WBA-UHT results.

In our study shelf life storage of 4 weeks for Pas and 24 weeks for UHT samples were chosen based on previous studies (Rustom, López-Leiva, & Nair, 1995; Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan, 2010).

Incorporation of additional well-established antioxidants to WB-Pas improved homogeneity of particle size distribution. Cao and Xiong (2017) observed interaction of whey proteins, particularly β -lactoglobulin and α -lactalbumin with EGCG at pH 3. Moreover, EGCG could induce conformational changes of β -lactoglobulin (Carnovale, Britten, Couillard, & Bazinet, 2015). These interactions may explain the observed modifications in particle size distribution of WBA-Pas. However, there is a discrepancy with our sedimentation analysis results, as WBA-Pas had significantly higher sedimentation than WB-Pas, requiring further investigation. From a processing perspective, high thermal loads and pH contributed to clarity of UHT whey protein beverages (Wagoner, Ward, & Foegeding, 2015). Moreover, heating and low pH reduced the particle size of our UHT beverages which increased stability. This is an expected result (Kubo, Augusto, & Cristianini, 2013) and underlines the improvement in product quality by UHT processing.

Conclusion

UHT processing increased the antioxidant activity of WB compared to pasteurization. Spray drying did not reduce antioxidant capacities of WB or WBA over a 24 week storage period. The hydrolytic conditions of the gut altered the antioxidant capacities of the beverages but this was dependent on processing type and antioxidant methodology. Incorporation of additional antioxidant ingredients in WB had no effect on apparent viscosity or instability index, but incorporation did increase sedimentation of pasteurised beverages, and redness and yellowness of both fresh pasteurised and UHT beverages. Moreover UHT treatment decreased instability index of beverages compared

to pasteurisation. In future, the capacity of these formulations to boost antioxidant defenses in cells and antioxidant biomarkers *in vivo* should be evaluated.

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Figure captions

Figure 1. Particle size distribution of pasteurized (Pas) (A) (liquid) and spray-dried powder (SD) (B) (solid) formulations measured by integrated light scattering. Measurements were performed in whey (WB) (*empty triangles*) and in whey with additional antioxidant ingredients (WBA) (*filled circles*) products.

Figure 2. Scanning electron micrographs of whey spray-dried powder formula (WB-SD) (A and B) and whey with additional antioxidant ingredients spray-dried powder formula (WBA-SD) (C and D). The scale bars represent 2 μm (A and C) and 10 μm (B and D).

Figure 3. Transmission profile of whey pasteurised (WB-Pas) (A), whey UHT (WB-UHT) (B), whey with additional antioxidant ingredients pasteurised (WBA-Pas) (C) and whey with additional antioxidants ingredients UHT (WBA-UHT) (D) formulations on day 1. Pasteurized and UHT samples transmission profiles simulating 20 days and 6 months of storage, respectively.

Figure 4. Antioxidant results of pasteurized (Pas), reconstituted spray-dried powder (SD) and UHT (UHT) products of whey beverage (WB) and whey with additional antioxidant ingredients (WBA) over time at standard conditions of storage. ABTS results (A and B) are expressed as % radical inhibition (absorbance control vehicle ABTS radical calculated as 100%), samples were diluted in reverse osmosis H_2O (1:3) and 5 μL of sample was tested. Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). FRAP results (C and D) are expressed as μmol Trolox Equivalents (TE) per 100 mL of beverage (calculated using a Trolox standard curve). Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). ORAC values (E and F) expressed as μmol TE per gram 100 mL of beverage (calculated using a Trolox standard curve). Results represent the mean of two experimental repetitions \pm standard deviation ($n = 6$). Samples with different letters are significantly different within each time point ($p < 0.05$). * indicates significant difference with time point 0 ($p < 0.05$).

Figure 5. Antioxidant results of fresh (time zero, black bars) and simulated gastrointestinal digested (grey bars) pasteurized (Pas), reconstituted spray-dried powder (SD) and UHT (UHT) products of whey beverage (WB) and whey with additional antioxidant ingredients (WBA). ABTS results (A) are expressed as % radical inhibition, samples were diluted 4 times in reverse osmosis H_2O and 5 μL of sample was tested. Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). FRAP results (B) are expressed as μmol Trolox Equivalents (TE) per 100 mL of beverage. Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). ORAC values (C) expressed as μmol TE per 100 mL of beverage. Results represent the mean of two experimental repetitions \pm standard deviation ($n = 6$). Samples with different letters are significantly different ($p < 0.05$).

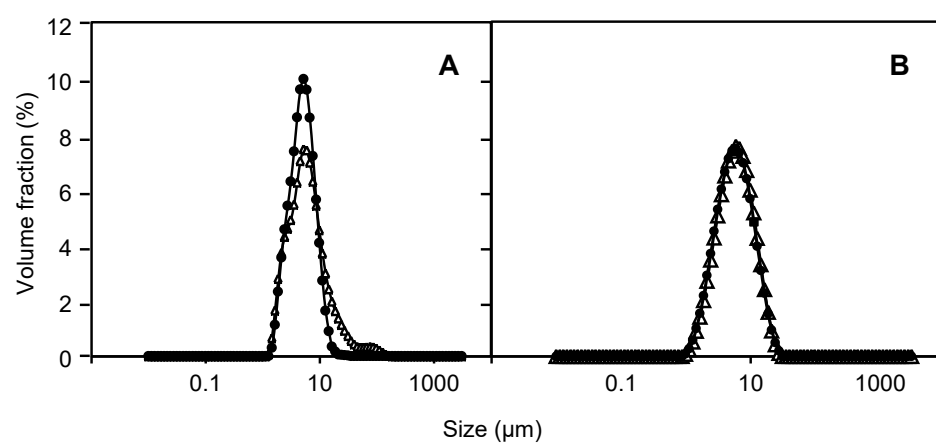
Figure 1.

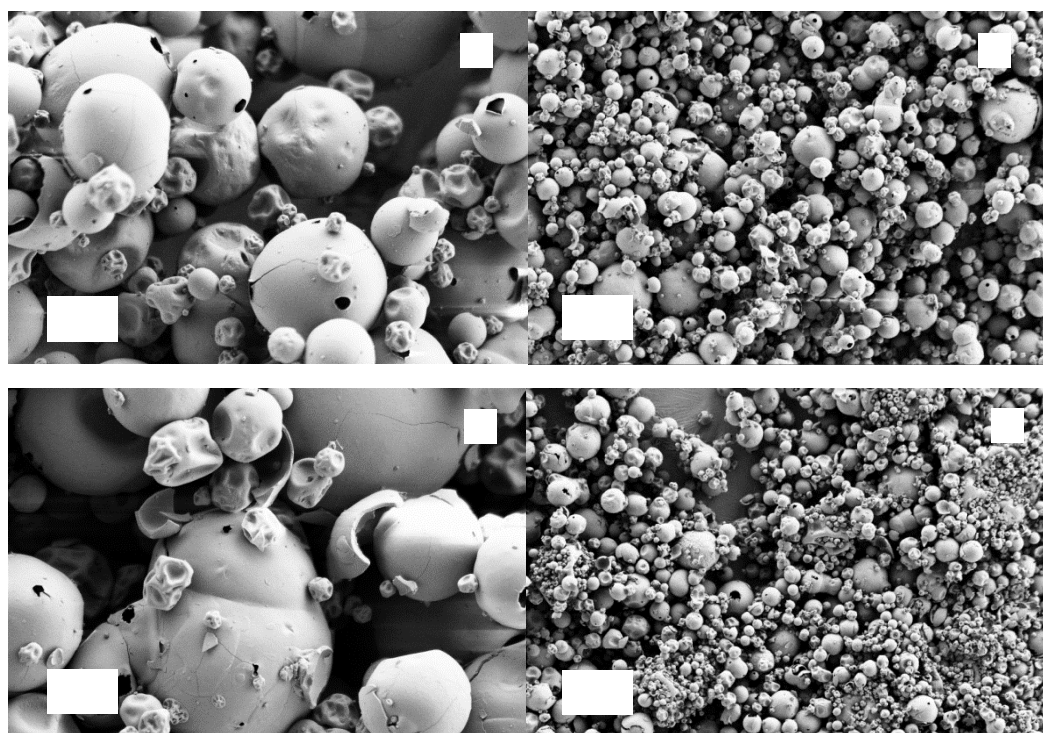
Figure 2.

Figure 3.

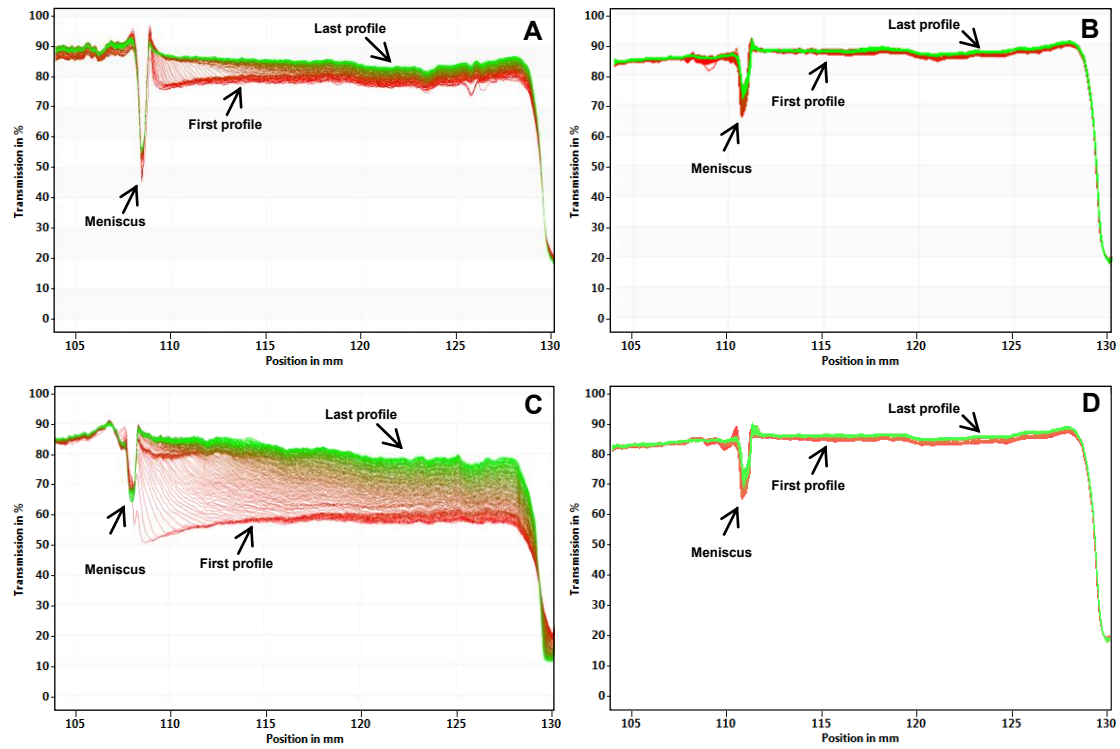
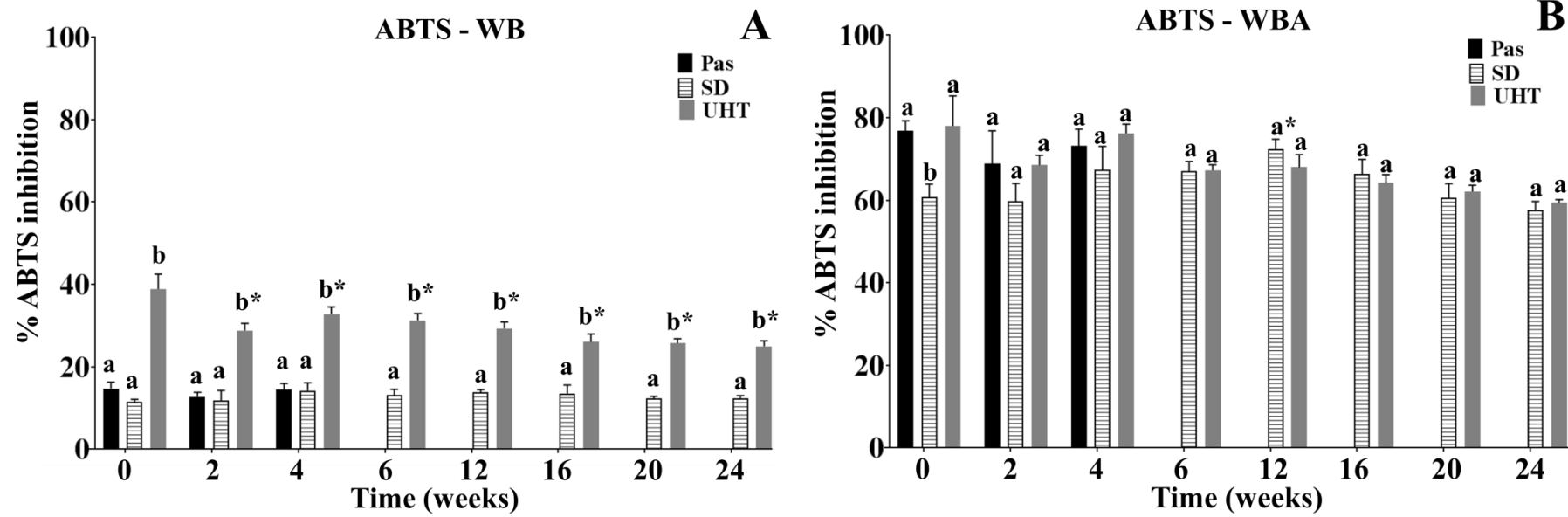
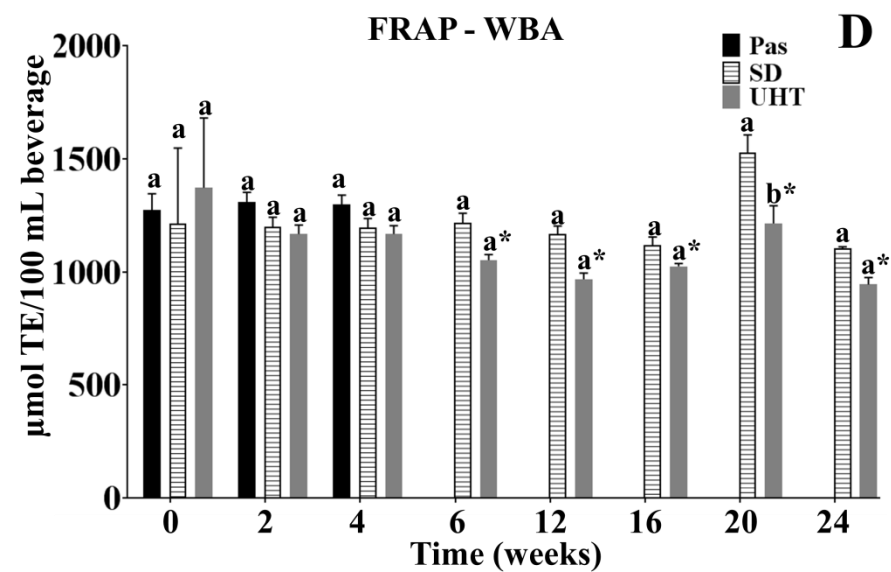
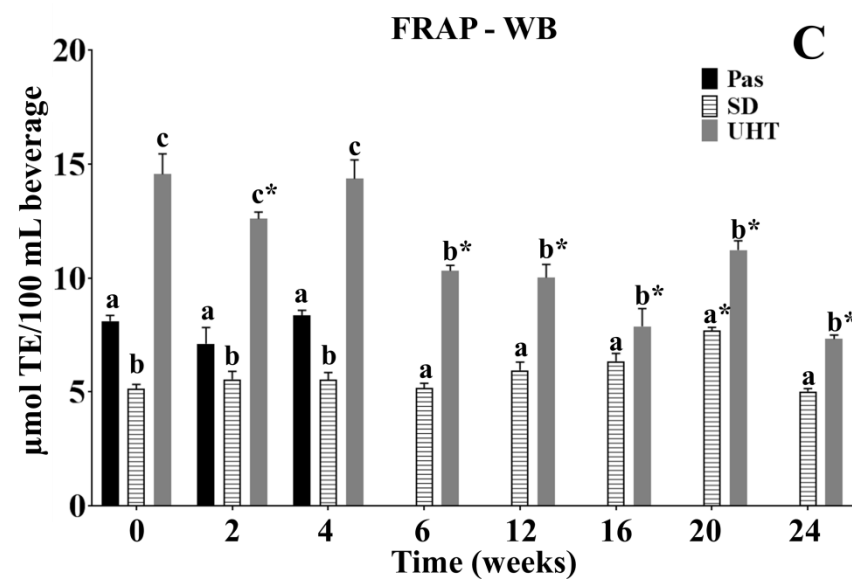


Figure 4.





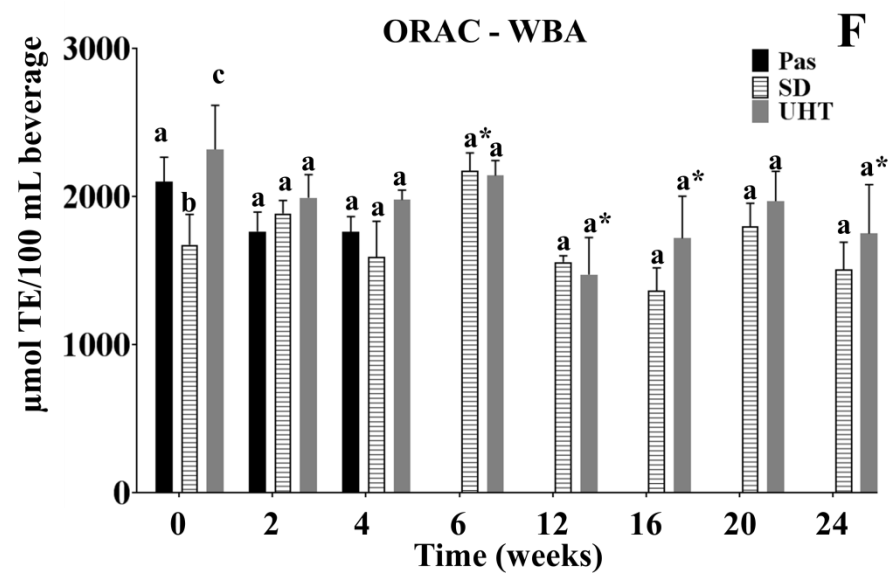
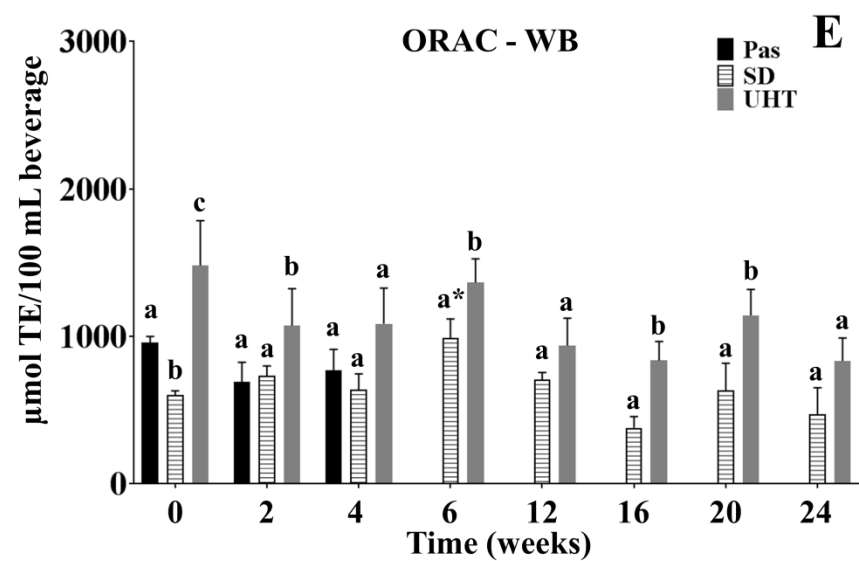
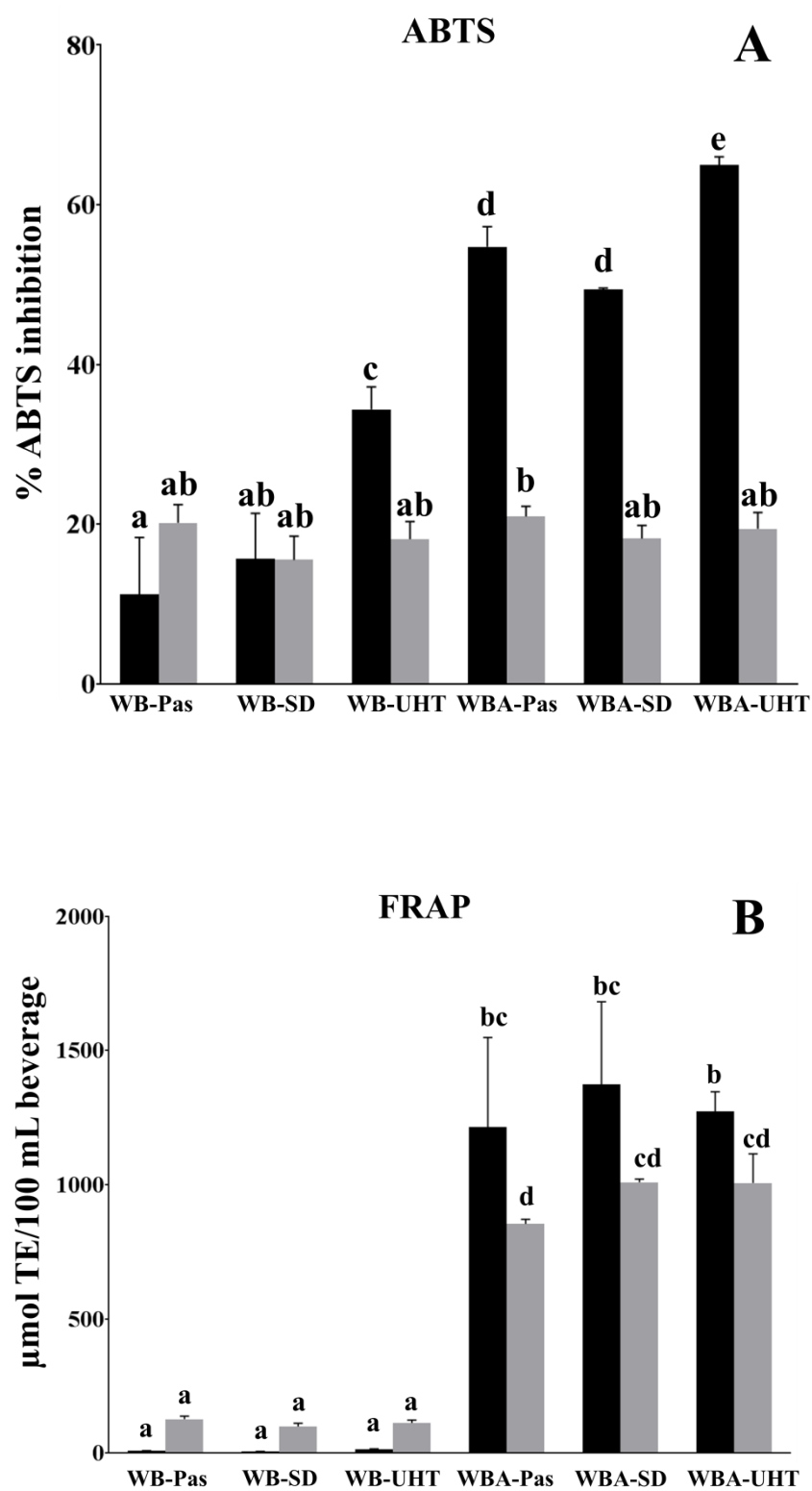
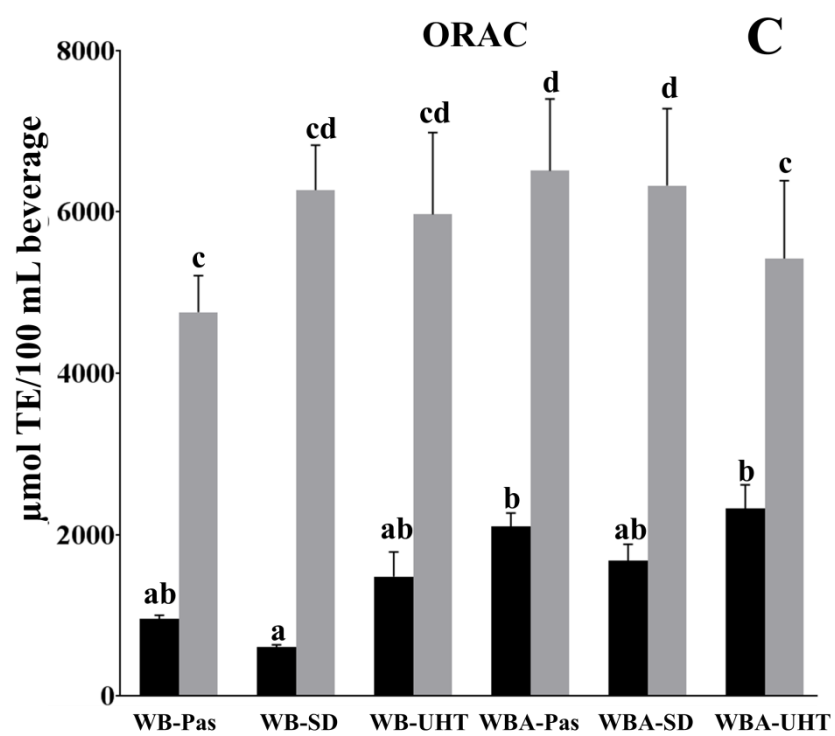


Figure 5.





Tables**Table 1.** Composition of whey (WB) and whey with additional antioxidant ingredients (WBA) beverages.

Ingredients	Concentration (% w/v)	
	WB	WBA
Reverse osmosis water	87.50	87.42
Maltodextrin	8.00	8.00
WPI	4.40	4.40
α -lactalbumin	0.10	0.10
Vitamin B12	-	7.20 E-07
Astaxanthin	-	7.20 E-03
EGCG	-	7.50 E-02
Marjoram extract	-	3.00 E-03

Table 2. Viscosity, instability index and sedimentation analysis (velocity) of pasteurized (Pas) and UHT (UHT) products obtained from whey (WB) and whey plus additional antioxidant ingredients (WBA) liquid beverages. Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). Samples with different letters are significantly different within each parameter (viscosity, instability index and velocity) ($p < 0.05$).

	Pas				UHT		
	Viscosity (mPa s)	Instability index		Velocity (mm day ⁻¹)	Viscosity (mPa s)	Instability index	
		20 days	6 months			20 days	6 months
WB	2.47 \pm 0.01 ^{ab}	0.53 \pm 0.01 ^a	0.63 \pm 0.02 ^{ab}	77.94 \pm 5.54 ^a	2.55 \pm 0.01 ^b	0.04 \pm 0.03 ^c	0.16 \pm 0.04 ^{cd}
WBA	2.54 \pm 0.03 ^b	0.71 \pm 0.04 ^{ab}	0.81 \pm 0.01 ^b	172.28 \pm 19.88 ^b	2.44 \pm 0.01 ^a	0.11 \pm 0.07 ^{cd}	0.26 \pm 0.06 ^d

Table 3. Colour of pasteurized (Pas), spray-dried powders (SD) and UHT (UHT) products obtained from whey beverage (WB) and whey plus additional antioxidant ingredients (WBA). L* indicates lightness, a* indicates redness/greenness and b* is yellowness/blueness. Differences in colour are shown between formulations (Δ Formulas (WBA vs WB)) and during storage at standard conditions of 4 weeks for Pas samples (Δ Formulas (0 vs 4 weeks)) and 24 weeks for SD and UHT samples (Δ Formulas (0 vs 24 weeks)). Total colour differences (ΔE) were calculated between formulations (ΔE (WBA vs WB)) and during storage at standard conditions of 4 weeks for Pas samples (ΔE (0 vs 4 weeks)) and 24 weeks for SD and UHT samples (ΔE (0 vs 24 weeks)). Results represent the mean of two experimental repetitions \pm standard deviation ($n = 4$). Different subscript lowercase letters represent significant difference in L*, a* or b* parameters within each beverage (SD products were

measured as powders) ($p < 0.05$). Different subscript uppercase letters represent significant differences in ΔE within each beverage (SD products were measured as powders) ($p < 0.05$).

		Pas				SD				UHT			
		WB	WBA	Δ Formulas (WB - WBA)	ΔE (WB - WBA)	WB	WBA	Δ Formulas (WB - WBA)	ΔE (WB - WBA)	WB	WBA	Δ Formulas (WB - WBA)	ΔE (WB - WBA)
0 weeks	L*	30.70±0.28 ^a	32.74±1.61 ^{ab}	-2.04±1.89		93.60±1.54 ^a	90.89±2.05 ^a	2.71±1.42		33.77±0.04 ^{ab}	31.95±0.06 ^{bc}	1.83±0.11	
	a*	-	-		2.56±1.43 ^{AB}	1.29±0.31 ^{ab}	3.47±0.55 ^b		4.03±1.67 ^A	-	-		4.04±0.05 ^A
	b*	0.13±0.06 ^a	0.97±0.16 ^b	-1.10±0.21		5.56±0.97 ^a	7.52±1.36 ^a	-2.19±0.35		0.21±0.01 ^a	1.66±0.03 ^b	-1.87±0.03	
4 weeks	L*	2.23±0.01 ^a	1.63±0.02 ^b	0.61±0.03				-1.96±1.08		1.27±0.06 ^a	4.35±0.09 ^b	-3.08±0.15	
	a*	36.48±0.06 ^b	36.67±0.44 ^b	-0.20±0.51		90.50±4.52 ^a	95.82±1.24 ^a	-5.32±2.64		37.36±0.07 ^a	34.20±0.06 ^{ab}	3.16±0.15	
	b*	0.18±0.03 ^a	1.10±0.11 ^b	-0.92±0.05	1.04±0.10 ^A	0.32±0.26 ^{ac}	3.24±0.85 ^b	-2.92±0.76	6.58±1.65 ^A	0.02±0.03 ^a	2.20±0.07 ^b	-2.21±0.02	4.75±0.01 ^A
24 weeks	L*	2.39±0.08 ^a	2.27±0.19 ^a	0.17±0.27		9.99±2.97 ^a	10.08±2.12 ^a	-0.09±2.81		2.20±0.11 ^c	5.16±0.02 ^b	-2.97±0.17	
	a*	-	-	-		96.75±1.23 ^a	95.46±1.11 ^a	1.29±2.74		29.91±1.18 ^c	24.66±1.48 ^d	5.25±0.05	
	b*	-	-	-	-	0.06±0.09 ^a	2.32±0.21 ^{bc}	-2.27±0.25	3.72±1.27 ^A	-	-		7.82±0.16 ^B
Δ Formulas (0 - 4 weeks)	L*	6.16±1.09 ^a	8.07±0.35 ^a	-1.91±0.93						0.44±0.03 ^a	3.23±0.40 ^c	-3.67±0.29	
	a*	-	-	-		3.10±1.95	4.93±2.11			3.01±0.35 ^c	7.50±0.55 ^d	-4.49±0.10	
	b*	-	-	-						-	-	-	-
	L*	5.78±0.32	3.93±0.97			0.97±0.08	0.24±1.18			-3.58±0.04	-2.25±0.01		
	a*	-	-	-	-					-	-	-	-
	b*	0.31±0.21	0.13±0.05							-0.19±0.02	-0.54±0.03		

	b *	- 0.16±0.08	- 0.64±0.25	-	-	- 4.43±0.94	- 2.56±3.32	-	-	- -0.93±0.95	- -0.08±0.97	-
Δ Formulas (0 - 24 weeks)	L *	-	-	-	-	3.15±1.47	4.57±0.15	-	-	3.86±0.79	7.29±0.73	-
	a *	-	-	-	-	1.24±0.01	1.16±0.59	-	-	0.23±0.02	-1.57±0.30	-
	b *	-	-	-	-	0.60±1.33	0.55±0.12	-	-	-1.74±0.38	-3.15±0.33	-
ΔE (0 - 4 weeks)		5.79±0.31 ^B	4.07±0.9%	-	-	5.55±1.86 ^A	6.27±0.35 ^A	-	-	3.67±0.06 ^{AC}	2.41±0.04 ^C	-
ΔE (0 - 24 weeks)		-	-	-	-	3.63±1.05 ^A	4.88±0.12 ^A	-	-	4.27±0.56 ^A	8.10±0.47 ^B	-

Highlights

- Marjoram polyphenols and astaxanthin were combined with whey proteins
- UHT processing produced clear and stable whey protein beverages
- Thermal processing and simulated digestion altered bioactivity of beverages
- Shelf life antioxidant activity of powder beverages was maintained during storage
- Spray drying did not alter the antioxidant potential of beverages

Industrial relevance

Whey protein-based antioxidant beverages could benefit the elderly consumer to meet their increased protein requirements and boost their antioxidant status. Consumer's acceptance for whey protein-based beverages often improves with clear formulations. This work generated whey protein-based UHT beverages with greater stability and clarity than pasteurised formulations. A novel combination of plant and marine antioxidants increased antioxidant activity of whey protein-based formulations. Furthermore, to suit export markets this work generated spray dried whey protein formulations that did not alter antioxidant potential