Received: 2 February 2020 | Revised: 29 June 2020 | Accepted: 15 July 2020

DOI: 10.1111/jfpp.14845

ORIGINAL ARTICLE

Journal of Food Processing and Preservation | Institute of Food Science + Technology | ifst | WILEY

Effect of ultra-high pressure homogenization combined with β-cyclodextrin in the development of a cholesterol-reduced whole milk

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Funding information
Agencia Nacional de Investigación e Innovación, Grant/Award Number: PR_FSA_200911539

Abstract

The aim of this work was to study the combined effect of ultra-high pressure homogenization (UHPH) and β-cyclodextrin (β-CD) on cholesterol removal from bovine raw milk. Whole raw milk (3.87% ± 0.49% fat) was submitted to UHPH (0, 100, 200, and 300 MPa) and the addition of β-CD (0%, 0.1%, 0.3%, and 0.6%). Cholesterol removal increased with β-CD concentration and pressure applied. For 0.6% β-CD and 100 MPa, cholesterol removal was 65%; the highest cholesterol removal was obtained for 0.6% β-CD and 200–300 MPa reaching 87%–89%. Aggregation phenomena caused by UHPH (300 MPa) did not affect complex formation, whereas β-CD binding increased above 0.3%. UHPH increased L^* and reduced b^* color parameters. UHPH reduced 3 log cfu/ml of total aerobic counts. Cholesterol reduced milk produced under selected conditions (200 MPa, 0.6% β-CD) was comparable in terms of consumer acceptance to commercial pasteurized or UHT milks.

Practical applications

This work obtained cholesterol-reduced milk using UHPH and the addition of β-CD. This combination of technologies achieves a decrease in the particle size of whole fat milk and a reduction in cholesterol of 87%–89% using low amounts of β-CD. Cholesterol free milk can be used in traditional, innovative, and functional dairy products to potentially contributing to manage the risk of cardiovascular diseases.

1 | INTRODUCTION

Cardiovascular diseases represent 31% of deaths worldwide (World Health Organization, WHO, 2017). Among the controllable risk factors (such as diet, physical inactivity, and smoking), a high cholesterol intake from animal fats has been associated with the development of coronary artery disease and hypertension (Oggioni et al., 2015). In bovine milk, cholesterol (3-hydroxy-5,6-cholestene; the major sterol in whole milk) is a nonpolar compound present in concentrations of 10 to 30 mg/dl (Jensen, 2002). Lipids of milk are secreted in the form of fat globules, which are enveloped by a biological membrane (milk fat globule membrane; MFGM). Cholesterol is transported in milk fat globules and associates easily with phospholipids of the MFGM (MacGibbon & Taylor, 2006). Milk fat globules contain components that have beneficial properties for health such vitamins, conjugated linoleic acid, phospholipids, and sphingolipids (Castro-Gómez, Garcia-Serrano, Visioli, & Fontecha, 2015; Ortega-Anaya & Jiménez-Flores, 2019; Siurana & Calsamiglia, 2016). Fats also play a key role in the formulation of many dairy products, since they contribute to the properties of texture, smell, and taste (Coppa et al., 2011; Logan et al., 2017; McGhee, Jones, & Park, 2015). Therefore, it is important to reduce cholesterol while maintaining other nutritive components of milk fat.

With a view to reducing health risk factors, cholesterol removal from milk and other foods has been studied (Gómez-Cortés, Viturro, Juárez, & De la Fuente, 2015; Jung, Ganesan, Lee, & Kwak, 2013; Sun, Yang, Zhong, Zhang, & Wang, 2011). Different methods to reduce

cholesterol from food matrixes have been studied: (a) physical methods, such as supercritical fluid extraction, distillation, and crystallization (Huber, Molero, Pereyra, & Martínez de la Ossa, 1996; Mohamed, Saldan, Socantaype, & Kieckbusch, 2000; Sahena et al., 2009); (b) biological methods using *Nocardia labegensis* and *Rhodococcus equi* (Sieber, Schobinger Rehberger, & Walther, 2011; Watanabe, Aihara, & Nakamura, 1989); and (c) chemical methods: solid-liquid, liquid-liquid extraction, etc. (Garcia Rojas, Coimbra, Minim, & Freitas, 2007) and the use of β-cyclodextrin (β-CD). β-CD is a cyclic oligosaccharide made up by α-D glucopyranose with α-(1–4) bonds resulting from the degradation of starch by bacteria such as *Bacillus macerans* (Astray, González-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara, 2009; Rinaldi, Binello, Stolle, Curini, & Cravotto, 2015). β-CD is a nontoxic, edible, nonhygroscopic compound, listed as a Generally Recognized as Safe (GRAS) substance by the Food and Drug Administration (FDA) and is accepted as food additive (Codex Alimentarius Commission, 1995). β-CD can form stable inclusion complexes with nonpolar compounds, such as cholesterol. The complex formed between cholesterol and β-CD is easily separated by centrifugation (Astray et al., 2009; Kwak, Kim, Kim, Choi, & Kang, 2004). The use of β-CD in homogenized reconstituted whole milk powder (Rozycki et al., 2013) was studied, reaching up to 92% of cholesterol removal using 1.5% β-CD; similar rates (up to 95%; 0.6% β-CD) were reported in pasteurized whole milk (Alonso, Cuesta, Fontecha, Juarez, & Gilliland, 2009). In order to reduce the amount of β-CD used and the residue resulting from the separation of the cholesterol-β-CD complex, other authors have studied the immobilization of β-CD on different surfaces (Kwak et al., 2004; Tahir & Lee, 2013; Tahir et al., 2013). Cholesterol reduced milk obtained using β-CD, was sensory evaluated by Rozycki et al. (2013); 77% of consumers rated it as good or very good in terms of level of acceptance.

Ultra-high pressure homogenization (UHPH) is an emerging technology gaining increasing interest due to its effectiveness for the inactivation of pathogenic and spoilage microorganisms without altering the organoleptic and/or nutritional characteristics of milk, juices, and beverages (Dumay et al., 2013; Georget, Miller, Callanan, Heinz, & Mathys, 2014; Sevenich & Mathys, 2018; Zamora & Guamis, 2015). Other benefits and functionalities offered by the application of this technology are related to the effect it produces on the different food components, particularly on proteins and fats, with the subsequent modification of the functional and structural properties of dairy products and other foods (Trujillo, Roig-Sagués, Zamora, & Ferragut, 2016; Zamora & Guamis, 2015). In conventional homogenization fluids are forced to pass through a gap of approximately 100–300 μm at pressures ranging from 10 to 60 MPa (Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003). UHPH is a similar process but with operating pressures up to 400 MPa when the fluid passes through a gap of 2.0–2.5 μm, in which a combination of physical phenomena occurs (as high pressure, shear stress, impingement, cavitation, shock waves, temperature increase, impact, and turbulence) and results in modified physicochemical properties of the processed fluid food and microbial inactivation (Dumay et al., 2013; Floury, Legrand, & Desrumaux, 2004). Pereda, Ferragut, Quevedo, Guamis, and Trujillo (2007) obtained a 3.3–3.5 log reduction of total bacteria, psychrotrophs, and lactobacilli in bovine milk, at pressures of 200 MP and 300 MPa and inlet temperatures of 30°C and 40 °C. UHPH reduces milk fat globule size to the nano-scale when pressures above 100 MPa are applied, thus, producing a greater number of fat globules and increasing their surface area (Hayes & Kelly, 2003; Zamora, Ferragut, Guamis, & Trujillo, 2012). In this process, the milk fat globule membrane is destroyed, thus increasing the surface exposed with the formation of a new fat globule membrane material (Zamora et al., 2012). Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, and Trujillo (2014) compared UHT milk with UHPH-treated milk (200–300 MPa, 55–85 °C inlet temperature) by means of a panel of judges trained in different flavor attributes, and did not find significant differences between both treatments in terms of color, appearance, and mouthfeel, except for the attribute of cooked flavor.

We hypothesized that processing milk by means of UHPH may result in greater cholesterol availability, improving in this way the interaction with the complexing agent (β-CD) and increasing removal efficiency. Thus, the aim of this work was to study the effect of UHPH and β-CD concentration on cholesterol removal from whole raw milk and to assess overall acceptance for selected treatment conditions.

2 | MATERIALS AND METHODS

2.1 | Experimental design

To perform cholesterol removal, a factorial design with the following two factors was used: a) homogenization pressures (0, 100, 200, and 300 MPa), b) β-CD concentration (0%, 0.1%, 0.3%, and 0.6%). Experiments were conducted in triplicate.

Cholesterol removal was assessed for all pressures and β-CD concentrations in order to select optimal processing conditions. Particle size and color analyses were performed for samples corresponding to optimal β-CD concentrations, where removal of cholesterol was higher.

Consumer acceptance and aerobic mesophilic bacteria counts were carried out in cholesterol-reduced milk obtained using the condition that presented the highest cholesterol removal and smallest particle size.

2.2 | UHPH treatment

Fresh raw bovine milk (3.87 ± 0.49% of fat and 332 ± 78 mg of cholesterol/100 g of milk fat) was collected from a dairy farm located in route 6, Sauce, Canelones, Uruguay. Milk was transported under refrigerating conditions (4–7 °C) in a previously washed and disinfected container. Upon arrival the container was stored in a cold chamber (4–7 °C), milk was used for experimental trials in less than an hour. Raw milk was preheated in a water bath (Haake D1, Germany) to 32 °C (inlet temperature, Tin). Then, milk at this temperature (Tin) was subjected to UHPH at 100, 200, and 300 MPa

using a two-stage continuous high-pressure valve homogenizer (model FPG 12,500, Stansted Fluid Power, Essex, UK). The pressure of the second valve was set to zero for all the experiments described in the present work. The temperature after the high pressure valve (Tv) was monitored throughout the UHPH process. The flow rate of milk in the homogenizer was approximately 8 L/h. UHPH processed samples (at 100, 200, and 300 MPa) were identified as HM100, HM200, and HM300, respectively. Nonhomogenized raw milk was identified as RM. Processed samples were collected into a sterile container and immediately placed on an ice bath cooled to 10 °C.

2.3 | Cholesterol removal

β-CD 99.8% purity (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to 100 ml of raw milk and of each homogenized milk (RM, HM100, HM200 and HM300) to obtain the different concentrations of β-CD (0%, 0.1%, 0.3%, and 0.6%) at 10 °C and was stirred at 530 rpm for 15 min. Then, samples were kept at 4 °C for 16 hr, they were centrifuged at 158 g for 10 min at 4 °C (Hael Force Centrifuge, Shanghai Lishen Scientific Equipment. Co. Ltd., China; Lee, Ahn, and Kwak (1999). For each treatment after centrifugation, the supernatant was separated, and the fraction containing cholesterol-reduced milk was used for cholesterol determination. Cholesterol reduced milks (CR) were identified as CRRM, CRHM100, CRHM200, and CRHM300 (for RM, HM100, HM200 and HM300, respectively).

2.4 | Cholesterol determination

Cholesterol analysis in milk was performed after each treatment as according to AOAC 994.10 "Cholesterol in Foods. Direct Saponification-Gas Chromatographic Method". Saponification was carried out using 50% KOH (Macron, Fine Chemicals, Mexico) and ethanol/methanol (95:5 vol/vol) (Burdick & Jackson, Honeywell International Inc., Charlotte, North Carolina, USA). An aliquot of the hexane (Macron, Fine Chemicals, USA) solution was brought to dryness and re-dissolved with dimethylformamide (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and finally, it was derivatized with hexamethyldisiloxane (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and trimethylchlorosilane (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). A 5 α-cholestane internal standard solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in heptane was used. A gas chromatographer-mass spectrometer with electron impact (Agilent MS 5975C Inert XL and Agilent GC 7890A) was employed. The following conditions were used: injection temperature 250 °C, splitless injection mode, initial oven temperature of 190 °C for 4 min, temperature ramp rate of 30 °C/min up to 280 °C, final oven temperature of 280 °C for 20 min. A capillary column was used: HP-5MS 0.25 mm × 30 m x 0.25 μm or similar, carrier gas helium (purity 99.999%), electron impact ionization mass detector and source temperature of 200 °C. All reagents used were of quality for residue analysis.

The percentage of cholesterol removal was calculated using the following equation:

$$\text{Cholestrol removal}\,(\%) = [Ci - Cf/Ci] \times 100$$

where: *Ci:* Concentration of cholesterol in initial raw milk. *Cf:* Concentration of cholesterol in milk upon finalization of removal process according to each treatment.

The percentage of complexed β-CD in milk after cholesterol removal was calculated as follows:

$$\%\text{complexed}\beta - \text{CD} = \text{complxed}\beta - \text{CDconcentration} * 100/\beta - \text{CDconcentrationadded}$$

The complexed β-CD concentration was considering the initial concentration of cholesterol in raw milk in each experiment and the removal percentages obtained in each treatment.

For calculations, the molecular weights of cholesterol and β-CD (387 g/mol and 1,135 g/mol, respectively) and stoichiometric ratio between complexed β-CD and cholesterol 3 to 1, were used (Rozycki et al., 2013).

2.5 | Particle size determination

The particle size determination of milk samples was carried out using a Microtrac S3500 laser diffraction particle size analyzer (Microtec. Inc., USA). It was performed in samples with no β-CD added and in the fraction containing cholesterol-reduced milk (supernatant) of milks added with 0.6% of β-CD. Samples were diluted with MilliQ water until an appropriate obscuration level was achieved for diffraction (40%). A refractive index of 1.46 (real) and 1.33 (water) was used. Measurements were carried out in triplicate, at room temperature of approximately 20 °C. Specific surface area values were calculated, and the size distribution was characterized by the diameter below which 50% or 90% of the volume of particles were found (D_{50}, D_{90}, respectively), the Sauter diameter (surface-weighted mean diameter, [$D_{3,2}$]) and the volume-weighted mean diameter value ($D_{4,3}$). Each sample was measured by triplicate.

2.6 | Color

Color values of milk samples were determined using a Hunter Lab colorimeter (LabScan XE, Hunter Associates Laboratory Inc., Reston, VA). The CIELAB method with a D65 light source and a 10° standard observer was used; calibration was performed with two standard plates: a white plate and a black plate. It was performed in samples with no β-CD added and in the fraction containing cholesterol-reduced milk (supernatant) of milks added with 0.6% β-CD. Samples were placed into a glass protected from exterior

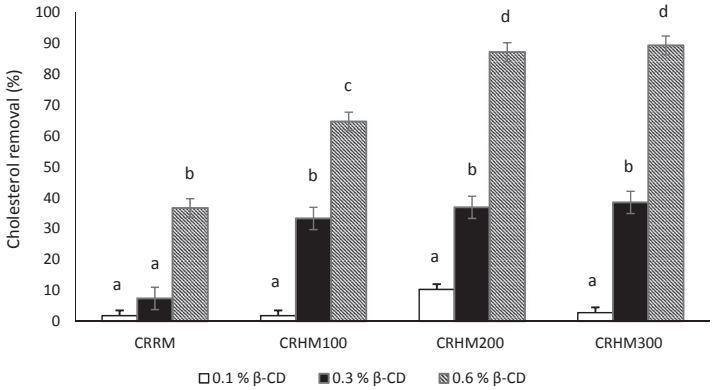

FIGURE 1 Cholesterol removal from bovine milk (wt/wt %) on the basis of raw milk (CRRM), UHPH milk at pressures of 100, 200, and 300 MPa (CRHM100, CRHM200, and CRHM300) and percentage of β-CD added (0.1% β-CD, 0.3% β-CD, and 0.6% β-CD). Mean values bearing the same letter are not significantly different from each other ($p > .05$); bars represent the standard error ($n = 6$)

light. L^* (luminosity) values ranging from 0 (black) to 100 (white) were measured. The values of a^* and b^* account for chromatic components. The positive values of a^* are red and the negative values are green, while the positive values of b^* are yellow and the negative values are blue.

2.7 | Total aerobic mesophilic count

Cholesterol-reduced UHPH milk (200 MPa, 0,6% β-CD), was maintained in the refrigerator in sterile container at 4 °C for 6 days. Samples were taken on days 1, 3, and 6 after elaboration.

Trials were conducted according to ISO 4833 "Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of microorganism- Colony-count technique at 30°C," using Milk Plate Count Agar as culture medium and incubating for 72 hr.

2.8 | Consumer acceptance

In order to determine consumer acceptance of the cholesterol-reduced milk developed and also to compare differences in terms of overall liking with respect to other milks in the market, a consumer acceptance test was performed. Milk samples were stored in the refrigerator at 40 °C for 24 hr, until their evaluation. Three samples were evaluated: the cholesterol-reduced milk developed in the present study (only the condition that presented the highest cholesterol removal and smallest particle size), commercial pasteurized whole milk (HTST, processed at 72 °C for 15 s) and ultra-high temperature whole milk (UHT, processed at 135 °C for 4 s) both from the same company of Uruguayan market. The three samples of the different milks were presented in glasses labeled with random 3-digit codes in a balanced presentation order for each participant to taste; no identification as to the type of milk of each sample was shown (blind samples). The evaluation was carried out in a standardized test room according to ISO 8,589:1988 under white artificial light. The evaluation was attended by 58 consumers aged 18–60, of which 49% were women. A 9-point structured Hedonic scale was used (1-Dislike extremely, 5-Neither like nor dislike, 9-Like extremely) to determine the degree of liking of samples (Drake, 2007).

2.9 | Statistical analysis

A three-way mixed-model ANOVA was used. β-CD percentages and milk homogenization pressures were treated as fixed factors and repetition was the random factor. The test was carried out using cholesterol concentration as covariate. Tukey's comparison tests with a 5% level of significance were performed. R Core Team (2013) was the software used.

3 | RESULTS AND DISCUSSION

3.1 | Cholesterol removal

As shown in Figure 1, cholesterol removal increased when β-CD concentration increased from 0.1% to 0.6% for raw milk (RM) and high-pressure homogenized milks. It was found that both the increase in β-CD concentration and in homogenization pressure had a significant impact on cholesterol removal ($p < .05$).

For RM, 7% of cholesterol was removed when 0.3% of β-CD was used, and increasing β-CD to 0.6% removed 37% of cholesterol. Similar removal values were obtained for UHPH-processed milk, using half the β-CD concentration (0.3%); for 100, 200, and 300 MPa, 33%, 37%, and 39% of cholesterol was removed, respectively.

When the concentration of β-CD increased to 0.6%, for CRHM100 samples cholesterol removal increased to 65%. The highest cholesterol removal rate was obtained using 0.6% of β-CD concentration and pressures of 200 and 300 MPa reaching 87% and 89%, respectively.

Cholesterol removal using β-CD has also been studied by other authors. Lee, Ahn, and Kwak (1999) have studied the conditions of the cholesterol removal process with β-CD as complexing agent, managing to remove up to 95% by using 1.5% β-CD concentration in commercial milk. Similar rates (~95%) were reported by Alonso et al. (2009) in pasteurized whole milk, allowing 20 min at 4 °C for β-CD-cholesterol complex formation, conditions which differ from those used in this study.

Rozycki et al. (2013) have studied the influence of conventional homogenization of milk in cholesterol removal working

with reconstituted whole milk powder. These authors have found a direct influence of homogenization pressures between 11.1 and 17.2 MPa, but they also stated that homogenization pressure had less influence than the percentage of β-CD used. They reported removal values of 83% and 92% for pressures of 135 atm (13.7 MPa), using 0.776% and 1.624% β-CD, respectively. Finally, they found that for reconstituted homogenized milk only 5 to 6% of the initial amount of β-CD added (1,624%) was binded to cholesterol.

In the present study, lower β-CD concentrations were chosen than the ones used in previous studies, hypothesizing that applying UHPH would originate higher percentages of removed cholesterol. Also, lower concentrations of β-CD might produce lower amounts of residual free β-CD after cholesterol removal. The percentage of added β-CD which formed the "cholesterol-β-CD" complex (% of complexed β-CD) was estimated (Table 1). Only 2 to 7% of the β-CD added to raw milk was estimated to form the "cholesterol-β-CD" complex. For cholesterol removed samples CRRM, CRHM100, CRHM200, and CRHM300, the addition of 0.6% β-CD produced higher percentages of complexed β-CD than the addition of 0.1% β-CD. These values of % complexed β-CD are higher than those found by Rozycki et al. (2013). This may be explained by a shifting balance in the formation of the "β-CD-cholesterol" complex as a result of increasing β-CD concentration. These results evidenced that the percentage of complexed β-CD may be affected not only by the concentration of β-CD added, but also by the pressures of homogenization.

Samples treated with 0.6% of β-CD and homogenization pressures of 200 and 300 MPa presented residual cholesterol concentrations in milk of 1.87 ± 0.61 and 1.65 ± 0.46 mg/100 g, respectively. According to the Guidelines for Use of Nutrition and Health Claims (Codex Alimentarius Commission, 1997), a food product is considered "free" of cholesterol when its concentration is lower than 5 mg/100 g. Therefore, milk obtained by UHPH above 200 MPa in compliance with these conditions can be called and marketed as "cholesterol-free milk."

The percentages of cholesterol removal obtained in the present work may seem similar or lower than the ones reported in previous studies. However, it must be kept in mind that in the present study fat content of raw milk was (3.87% ± 0.49%). Relating the concentration of 0.6% β-CD to milk fat content (0.6% β-CD/fat %), the ratios would be 0.6/3.87 (15.5%) and 0.6/3.0 (20.0%), for the raw milk used in the present study and for 3.0% fat milk, respectively. This ratio must also be taken into account when comparing cholesterol removal.

TABLE 1 % complexed β-CD (i.e., the percentage of β-CD added that was used to form the cholesterol-β-CD complex) on the basis of raw milk (CRRM) and UHPH milk (CRHM100, CRHM200, and CRHM300) and percentage of β-CD added (0.1% β-CD, 0.3% β-CD, and 0.6% β-CD)

	% complexed β-CD % β-CD added to milk		
	0.1%β-CD	0.3%β-CD	0.6%β-CD
CRRM	2.1 ± 1.9^{a*}	3.3 ± 1.7^{a}	7.2 ± 2.2^{b}
CRHM100	1.8 ± 2.8^{a}	10.9 ± 1.9^{bc}	11.7 ± 1.2^{bc}
CRHM200	10.4 ± 1.2^{bc}	14.2 ± 2.9^{bcd}	17.3 ± 0.4^{d}
CRHM300	3.1 ± 2.0^{a}	16.5 ± 2.4^{cd}	19.5 ± 2.5^{d}

*a–d Different superscripts indicate significant differences ($p < .05$). Values are means ± standard error of triplicate analysis. ($n = 6$).

3.2 | Particle size determination

The particle size distribution of RM samples presented a main peak at 4.4 μm and a small shoulder around 2 μm. After processing the samples with UHPH, exposing them to β-CD and removing cholesterol, significant ($p < .05$) changes in particle size distribution were observed. When treatments of 200 MPa were applied, the main peak shifted to 0.6 μm. Particle size parameters ($D_{3,2}$, $D_{4,3}$, D_{50}, and D_{90}) decreased with homogenization pressure up to 200 MPa, where it reached its minimum value, and then increased for samples processed at 300 MPa (Table 2).

Other authors have also registered size reduction with subsequent increase with homogenization pressure (Datta, Hayes, Deeth, & Kelly, 2005; Hayes & Kelly, 2003; Pereda et al., 2007; Thiebaud et al., 2003). Dumay et al. (2013) have also explained that there is a gradual but significant shift in terms of particle size distribution toward lower values when pressure increases; however, when pressure reaches 250/300 MPa, there is an increase due to particle aggregation.

In the present study, smaller particle sizes were found for samples treated at 300 MPa using 0.6% β-CD, than samples homogenized at the same pressure without the addition of β-CD (Table 2). These results would indicate that the presence of 0.6% β-CD could influence the different phenomena resulting from fat globule and protein aggregation. Aggregation was also observed by Zamora, Ferragut, Jaramillo, Guamis, and Trujillo (2007) by means of confocal laser scanning microscopy of rennet gels which revealed that this phenomenon was due to the aggregation of well-defined small fat globules within dense proteinaceous structures which explained their broader size distributions observed in milks treated at 300 MPa (first stage) and 30 MPa (second stage).

For UHPH milk processing, different authors have reported the association of proteins in the MFGM, such as β-Lg via disulfide bonds (Singh, 2006), casein micelles via disulfide bonds of κ-CN and also adsorption of "intact" casein micelles (Ye, Anema, & Singh, 2008). Zamora et al. (2012) have demonstrated the different associations taking place between membrane material and the different milk proteins during pasteurization, conventional homogenization, and high-pressure homogenization processes.

Dumay et al. (2013) established that heating mainly takes place for small fractions of time (<1 s), at an estimated speed of 200–250 m/s. Nevertheless, Dumay et al. (2013) state that aggregation can be the result of different phenomena, one of them being insufficient cooling immediately after the high-pressure valve (thus, favoring

TABLE 2 Particle size parameters (D_{50}, D_{90}, $D_{4,3}$, and $D_{3,2}$†) of raw milk and UHPH milk, without β-CD (0%) (RM, HM100, HM200, and HM300,) and with 0.6% β-CD (CRRM, CRHM100, CRHM200, and CRHM300)

Treated milk	β-CD	D_{50} (μm)	D_{90} (μm)	$D_{4,3}$(μm)	$D_{3,2}$(μm)	SSA (m^2/cm^3)
RM	0	$3.99 \pm 0.21^{c*}$	6.8 ± 1.06^{b}	4.31 ± 0.46^{c}	3.44 ± 0.05^{e}	1.75 ± 0.33^{a}
CRRM	0.6	4.23 ± 0.21^{c}	7.7 ± 1.06^{b}	5.29 ± 0.46^{c}	3.60 ± 0.05^{e}	1.67 ± 0.33^{a}
HM100	0	1.17 ± 0.21^{a}	6.1 ± 1.06^{b}	2.51 ± 0.46^{b}	0.98 ± 0.05^{b}	6.61 ± 0.33^{d}
CRHM100	0.6	1.31 ± 0.21^{a}	6.9 ± 1.06^{b}	2.84 ± 0.46^{b}	1.06 ± 0.05^{b}	5.66 ± 0.33^{cd}
HM200	0	0.63 ± 0.21^{a}	3.0 ± 1.06^{a}	1.31 ± 0.46^{a}	0.56 ± 0.05^{a}	10.81 ± 0.33^{f}
CRHM200	0.6	0.76 ± 0.21^{a}	5.7 ± 1.06^{b}	2.29 ± 0.46^{b}	0.68 ± 0.05^{a}	8.85 ± 0.33^{e}
HM300	0	4.42 ± 0.21^{c}	16.2 ± 1.06^{d}	6.92 ± 0.46^{d}	1.85 ± 0.05^{d}	3.25 ± 0.33^{ab}
CRHM300	0.6	2.60 ± 0.21^{b}	11.9 ± 1.06^{c}	4.97 ± 0.46^{c}	1.42 ± 0.05^{c}	4.24 ± 0.33^{bc}

*a–f Different superscripts in the same column indicate significant differences ($p < .05$). Values are means ± standard error of triplicate analysis ($n = 6$).

†D_{50}, diameter below which 50% of the volume of particles are found; D_{90}, diameter below which 90% of the volume of particles are found; $D_{3,2}$, Sauter diameter (surface-weighted mean diameter); $D_{4,3}$, volume weighted mean diameter, specific surface area (SSA).

TABLE 3 Parameters L^*, a^*, and b^* of raw milk and UHPH milk, without β-CD (0%) (RM, HM100, HM200, HM300,) and with 0.6% β-CD (CRRM, CRHM100, CRHM200, and CRHM300)

Treated milk	β-CD	L^*	a^*	b^*
RM	0	$91.61 \pm 0.08^{c*}$	-1.50 ± 0.09^{a}	14.21 ± 0.14^{a}
CRRM	0.6	92.29 ± 0.09^{c}	-1.43 ± 0.11^{a}	14.36 ± 0.18^{a}
HM100	0	94.17 ± 0.09^{b}	-0.81 ± 0.11^{c}	12.38 ± 0.18^{b}
CRHM100	0.6	94.18 ± 0.07^{b}	$- 0.87 \pm 0.08^{c}$	12.41 ± 0.13^{b}
HM200	0	94.40 ± 0.11^{ab}	-1.09 ± 0.07^{b}	12.20 ± 0.09^{bc}
CRHM200	0.6	94.51 ± 0.06^{ab}	-1.11 ± 0.08^{b}	12.36 ± 0.18^{b}c
HM300	0	94.66 ± 0.09^{ab}	-1.15 ± 0.11^{a}b	12.58 ± 0.18^{b}
CRHM300	0.6	94.74 ± 0.06^{a}	-1.43 ± 0.07^{a}	12.46 ± 12.08^{b}

*a–c Different superscripts in the same column indicate significant differences ($p < .05$). Values are means ± standard error of triplicate analysis ($n = 9$).

the denaturation of proteins and aiding the stabilization of fat). The temperature after the homogenization valve in samples treated at pressures of 100, 200, and 300 MPa, with an inlet temperature of 32 °C were: 56.5 ± 1.8 °C; 70.7 ± 2.3 °C and 93.5 ± 3.6 °C, respectively. Similar increases were reported by other authors, who have shown that a linear temperature increase takes place at homogenization valves where the mechanical and kinetic energy transforms into thermal energy (Donsi, Ferrari, Lenzab, & Maresca, 2009; Hayes & Kelly, 2003; Pinho, Franchi, Tribst, & Cristianini, 2011).

In the present study, the specific surface area proved to be higher when homogenization was performed at 200 MPa rather than at 300 MPa, being particle size related to this (Table 2). The highest cholesterol removal rates (Figure 1) were obtained when homogenization was performed at 200 and 300 MPa and were not statistically different (87 and 89%; $p > .05$). This could be ascribed to the fact that, after the rupture of the MFGM, cholesterol is released and is available for complex formation, regardless of the subsequent changes in the newly generated membrane and aggregation phenomena. Supposing that all β-CD binded to cholesterol is removed effectively, there were found higher β-CD-complexed values for 0.6 than for 0.1% added β-CD. This might indicate that in order to increase binding efficiency, β-CD concentration must be added above 0.3% (taking into account the range of β-CD used in the present work).

3.3 | Color

The addition of β-CD had no effect on L^* values in raw or homogenized milks. Milks treated with UHPH presented higher L^* values than raw milk, and this increased with homogenization pressure (Table 3). The increased L^* values found in high-pressure homogenization milks have also been observed in other studies (Hayes & Kelly, 2003; Hayes, Fox, & Kelly, 2005; Pereda et al., 2007). These authors have suggested that this phenomenon is due to an increase in the number of particles of smaller size, which can diffract light more effectively, being milk thus perceived as whiter.

The parameter a^*, which describes the red-green color range, had no significant differences ($p > .05$) for raw milk and milk homogenized at 300 MPa. Milks treated at 100 and 200 MPa presented significantly higher values than those of raw milk ($p < .05$). As seen in Table 3, raw milk presents higher b* values than UHPH milks for all pressures studied. The same trend was noticed by Pereda et al. (2007) using homogenization pressures at 200 and 300 MPa,

with inlet temperatures of 30 and 40 °C; small changes in a* and b* were also mentioned by Hayes et al. (2005). The use of β-CD did not alter this trend, regardless of the concentration. With respect to *a** parameters, Hayes and Kelly (2003) also found less negative values using pressures of 100, 150, and 200 MPa, while according to Pereda et al., (2007), the trend was opposite, although they found significant differences in *a** values. On the contrary, Amador-Espejo et al. (2014), using pressures at 200 and 300 MPa and inlet temperatures between 55 and 85 °C, did not find significant changes in *a** and *b** under all the conditions studied.

Taking into account the results for cholesterol removal and particle size, UHPH at 200 MPa and addition of 0.6% β-CD were selected for consumer acceptance and microbiological evaluation, as optimal conditions.

3.4 | Microbiological evaluation

Total aerobic mesophilic counts were performed prior to consumer acceptance in order to study the reductions caused by UHPH processing conditions and run a safe sensory evaluation. In raw milk total aerobic mesophilic count was 5.2 ± 0.2 (log cfu/ml), and after applying the combined process (200 MPa and 0.6% β-CD), it was 2.1 ± 0.1 (log cfu/ml) in cholesterol-reduced milk. This reduction remained stable during 6 days, stored at 4 °C. Pereda et al., (2007) obtained 3-log reductions in psychrotrophic and coliform bacteria for 200 MPa and an inlet temperature of 30°C. Under similar pressure and inlet temperature conditions, several authors reported reductions of spoilage and pathogenic bacteria in milk (Hayes, Fox, & Kelly, 2005; Pedras, Pinho, Tribst, Franchi, & Cristianini, 2012; Ruiz-Espinosa et al., 2012).

3.5 | Consumer acceptance

The cholesterol-reduced whole milk (200 MPa and 0.6% β-CD concentration, 3.87% ± 0.49% fat) had the same acceptance level as commercial UHT milk (135 °C for 4 s, 3.0% fat) and high temperature short time pasteurization (HTST, 72 °C for 15 s, 2.6% fat) in the study of consumer acceptance. The acceptance levels obtained in this study for the three types of milk (measured by overall liking with a 6–7 rating on the hedonic scale) did not significantly differ ($p > .05$); they were: 6.8 ± 1.1 for HTST milk, 6.0 ± 1.5 for UHT milk, and 6.6 ± 1.4 for UHPH reduced-cholesterol milk. They are all in the range found by other authors for pasteurized milks evaluated at different processing conditions (Gandy et al., 2008; Potts, Amin, & Duncan, 2017).

This result would be indicating that the two processes used to obtain "cholesterol-free" whole milk had no effect on consumer acceptance. In the present study, consumers were not aware that one of the milk samples was cholesterol-reduced. It must be taken into consideration that the fat content of reduced cholesterol milk was 3.87% and this might have helped the scores for this milk.

4 | CONCLUSIONS

In the present study, bovine milk with 89% of cholesterol removed was obtained from raw milk (3.87% ± 0.49% fat) by applying UHPH processing (200 MPa, Tin 32 °C), addition of low levels of β-CD (0.6% in milk or 15.5 g β-CD/100 g fat) and centrifugation at 158 g to remove the complex β-CD-cholesterol. The milk produced under these conditions was comparable in terms of consumer acceptance to commercial pasteurized or UHT milks. Aggregation phenomena caused by UHPH did not impair complex formation whereas binding was increased above a threshold concentration of β-CD (i.e. 0.3%). Prior to the experiments undertaken in the present study, a hypothesis was made: the use of UHPH would increase cholesterol removal compared to previous studies with conventional homogenization. Conversely, the values obtained were similar to the removals generated using conventional homogenization, with the exception that raw milk used had higher fat content than standardized milks, and the concentration of β-CD used was lower. In future studies, it would be interesting to explore: the temperature and time of reaction between cholesterol and β-CD; the effect of fat content in β-CD cholesterol extraction; and the impact of centrifugation at higher g force. In addition, it would also be advisable to focus on possible interactions between β-CD and other compounds of milk (such vitamins A and D3 and long-chain fatty acids) and quantification of residual levels. The use of cholesterol free milk as an ingredient for dairy products (such as cheese, cream, or butter) might be of industrial relevance. Nevertheless, it must be borne in mind that cholesterol intake restrictions have been attenuated or eliminated from nutritional guidelines and that scientific discussions and controversy over cholesterol intake is still active.

ACKNOWLEDGMENTS

The authors acknowledge Laboratorio Tecnológico del Uruguay (LATU) and Agencia Nacional de Investigación e Innovación (ANII), Uruguay for financial support through the projects PR_FSA_200911539, and Departamento de Microbiología and Departamento de Análisis de Productos Agropecuarios del Laboratorio Tecnológico del Uruguay (LATU) for technical assistance.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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How to cite this article: Escobar Gianni D, Jorcin S, Lema P, Olazabal L, Medrano A, Lopez-Pedemonte T. Effect of ultra-high pressure homogenization combined with β-cyclodextrin in the development of a cholesterol-reduced whole milk. *J Food Process Preserv.* 2020;00:e14845. https://doi.org/10.1111/jfpp.14845