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ABSTRACT

Infant formula (IF) is submitted to several heat treatments during production, which can lead to denaturation or aggregation of proteins and promote Maillard reaction. The objective of this study was to investigate innovative minimal processing routes for the production of first-age IF powder, thus ensuring microbial safety with minimal level of protein denaturation. Three nutritionally complete IF powders were produced at a semi-industrial scale based on ingredients obtained by fresh bovine milk microfiltration (0.8 and 0.1-µm pore size membranes). Low-temperature vacuum evaporation (50°C) and spray-drying (inlet and outlet temperatures of 160 and 70°C, respectively) were conducted to produce the T− formula with no additional heat treatment. The T+ formula was produced with a moderate heat treatment (75°C for 2 min) applied before spray-drying, whereas the T+++ formula received successive heat treatments (72°C for 30 s on the milk; 90°C for 2–3 s before evaporation; 85°C for 2 min before spray-drying), thus mimicking commercial powdered IF. Protein denaturation and Maillard reaction products were followed throughout the production steps and the physicochemical properties of the powders were characterized. The 3 IF powders presented satisfactory physical properties in terms of a_w, free fat content, glass transition temperature, and solubility index, as well as satisfactory bacteriological quality with a total flora $<10^3$ cfu/g and an absence of pathogens when a high level of bacteriological quality of the ingredients was ensured. Protein denaturation occurred mostly during the heat treatments of T+ and T+++ and was limited during the spray-drying process. The IF powder produced without heat treatment (T-) presented a protein denaturation extent (6 ± 4%) significantly lower than that in T+++ (58 ± 0%), but not significantly different from that in T+ (10 ± 4%). Although T− tended to contain less Maillard reaction products than T+ and T+++, the Maillard reaction products did not significantly discriminate the infant formulas in the frame of this work. The present study demonstrated the feasibility of producing at a semi-industrial scale an infant formula being bacteriologically safe and containing a high content of native proteins. Application of a moderate heat treatment before spray-drying could further guarantee the microbiological quality of the IF powders while maintaining a low protein denaturation extent. This study opens up new avenues for the production of minimally processed IF powders.

Key words: infant formula, filtration, minimally processed, protein denaturation

INTRODUCTION

According to UNICEF (2020), 56% of infants 0 to 5 mo of age receive a human milk substitute, which is in most circumstances an infant formula (**IF**), most often available in a powder form. The IF powders are generally manufactured by blending skim milk with other dried ingredients, including whey proteins (**WP**) originating from cheese whey or less commonly from skim milk microfiltration (**MF**) dried permeate, which are all together rehydrated and subjected to a succession of thermal processes (pasteurization, evaporation, drying) to ensure the microbial safety and shelf life stability of the product. These successive heat treatments induce numerous physicochemical modifications of the components, among them the denaturation or aggregation of WP (Brodkorb et al., 2016). The latter can affect WP functionality during IF processing, in particular when they form aggregates that increase viscosity, reduce the emulsion stability, and decrease the overall processing performance (Joyce et al., 2017). In addition, the heat-induced protein denaturation may have nutritional consequences. It has been previously reported to affect the in vitro digestion kinetics of caseins (**CN;** Dupont et al., 2010; Halabi et al., 2020a) and of β-LG (Takagi

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et al., 2003; Peram et al., 2013), as well as the microstructure of the gastric digesta (Halabi et al., 2020a). It could further affect the dietary nitrogen retention by the body, such as reported in adults fed UHT-treated milk versus pasteurized or microfiltered milk (Lacroix et al., 2008). In addition, the heat-induced protein denaturation can modulate the protein allergenicity, by either masking or, on the contrary, exposing epitopes, depending on the intensity of the heat treatment (Golkar et al., 2019). However, some authors have reported an increased allergic sensitization in pasteurized versus raw milk (Roth-Walter et al., 2008) and a loss of immunologically active WP due their denaturation, thus decreasing the allergy-protective capacity of raw cow's milk (Abbring et al., 2020). This can be further modulated by the Maillard reaction products, resulting from the reaction between lysine-rich proteins and reducing sugar (lactose) induced by heat treatment. This results in the formation in the first stage of Amadori products, such as lactulosyllysine, up to more advanced glycation end products, such as N^{ε}-carboxymethyl-lysine (**CML**). Consequently, the lysine bioavailability can be affected (Nunes et al., 2019), in addition to other potential health effects, such as pro-inflammatory and pro-oxidant effects for CML (Elmhiri et al., 2015) and the promotion of allergies in infants (Baskara et al., 2017). Overall, maintaining as much as possible the proteins in their native state may improve the nutritional quality of IF, even though bovine and human WP differ by nature.

Integrating some MF steps to fresh milk into the IF production process could limit the heat treatment effect on IF constituents while maintaining the bacteriological safety, and is to date an understudied strategy in the area of IF production. Several studies have demonstrated that MF with 1.4-μm pore size ceramic membranes can substantially retain bacteria in skim milk, with an overall decimal reduction above 4 $\log_{10}$ units (Trouvé et al., 1991; Fauquant et al., 2009, 2014; Tomasula et al., 2011; Schmidt et al., 2012). More specifically, Madec et al. (1992) have reported a decimal reduction around 2 $\log_{10}$ units for *Listeria* and *Salmonella*, respectively, using nonvirulent strains, and Trouvé et al. (1991) have shown a decimal reduction close to 3 $\log_{10}$ units for bacteria with various morphologies and cellular volumes, representative of raw milk contamination. Therefore, MF appears as an alternative to usually applied thermal processes such as pasteurization for reducing microbial load of fresh milk, while maintaining proteins in their native state. In addition, MF with 0.1-μm pore size membrane can be used to separate CN micelles from the other components of smaller molecular weight (WP, lactose, vitamins, and minerals; Gésan-Guiziou et al., 1999; Saboya and Maubois, 2000). After concentration by ultrafiltration, the liquid WP-rich permeate can be mixed with the microfiltered milk to achieve the human milk protein CN:WP ratio (40:60), while ensuring bacteriological safety without heat treatments. Notwithstanding significant development of filtration in the dairy industry over the past decade, few studies have used its potential for limiting thermal processes, and thus, maintaining proteins in their native state in IF powder. The main challenge is to integrate such nonthermal process in an overall low temperature processing route, allowing the production of a first-age (0–6 mo) IF powder.

The objective of this study was to investigate whether a minimal processing route, based on filtration of fresh milk and other unit operations (evaporation, homogenization, spray-drying), could allow the production, at a semi-industrial scale, of a qualitative IF powder presenting a limited level of protein denaturation and being bacteriologically safe. The microfiltration for skim milk debacterization was decreased to a pore size of 0.8 μm to improve the bacteria retention (Tomasula et al., 2011), particularly, regarding *Cronobacter spp.* Three IF powders were produced: T−, without heat treatment; T+, with pasteurization of the concentrate alone (75°C for 2 min); T+++, with successive heat treatments including one on the concentrate at 85°C for 2 min before spray-drying. The extent of WP denaturation and the formation of Maillard reaction products were evaluated throughout the process. The physicochemical properties of IF powders before and after reconstitution were characterized. To the best of our knowledge, this is the first time that such a minimal processing technological route, integrating membrane filtration and spray-drying operations, is proposed and characterized for the production of a first-age IF.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, chemicals are from commercial origin (Sigma-Aldrich).

IF Ingredients

Raw cow milk (900 kg) was purchased from a local dairy cooperative (SODIAAL Entremont). Liquid WP isolate (**WPI**), obtained by low temperature (<10°C) MF 0.1 μm of fresh skim milk, followed by ultrafiltration, was purchased from a dairy company (confidential source). The WPI had a DM of 26.6% (wt/wt) of which 92% were proteins. The WP accounted for 93% (wt/wt) of the protein fraction, such as determined by the Kjeldahl method. Lactose was supplied by Lactalis In-

gredients. Oil blend and vitamin and mineral premixes, adapted to IF, were purchased from Cargill (Schiphol) and Firmalis (Levallois-Perret), respectively.

IF Processing

Three types of IF powders, namely T−, T+, and T+++ according to the heat treatments received during manufacture, were produced according to Figure 1. Fresh raw whole milk was skimmed by centrifugal cream separation at 50°C in rising phase and then subjected to MF using 0.8-μm pore size graded permeability (**GP**) Pall mineral membranes (Pall Corporation, Bazet, France) with a total filtration area of 1.68 m^2. The observed retention of total proteins, as well as of caseins, was 97.5%. The crossflow velocity was maintained at 7 $m \cdot s^{-1}$ (retentate flowrate of 43 $m^3 \cdot h^{-1}$) and the retentate pressure drop was maintained constant at 2 bar. The temperature was maintained at 50 ± 2°C with continuous cooling of the filtrate (microfiltered skim milk, **MSM**) using plate heat exchangers. The filtrate and the retentate fluxes were adjusted to 630 L/h (375 $L \cdot h^{-1}/m$) and 33 L/h, respectively, to maintain a volume concentration factor of 20. For T−, the MSM was directly subjected to the mixing step. For T+, the MSM was stored overnight at 4°C before mixing. For T+++, the MSM was pasteurized at 72°C for 30 s, stored overnight at 4°C, and repasteurized at 72°C for 30 s before mixing. For the mixing step, MSM, WPI (with a DM of 26.6% wt/wt), lactose [solubilized in sterilized osmosis water (130°C, 20 s) at 50% (wt/wt) and heated at 60°C] and minerals were mixed at 50°C under stirring. The mixed solutions were then concentrated to approximately 40% (wt/wt) of DM in a 2-stage semi-industrial scale falling film vacuum evaporator (GEA Process Engineering) with an evaporation capacity of 270 $L \cdot h^{-1}$ at 50°C. For T+++, the mixed solution received a heat treatment at 90°C for 2 to 3 s before concentration. After oil blend and vitamin addition at 50°C, and pH adjustment to 6.6 ± 0.2 (KOH, 45% wt/wt), the mixes were homogenized (50°C, 140/40 bar). For T−, the homogenized concentrate was directly spray-dried using a semi-industrial-scale 2-stage spray-dryer (Niro Atomizer, GEA-PE, Saint Quentin en Yvelines, France) at Bionov (Rennes, France), for which maximum theoretical evaporation capacity is approximately 90 $kg \cdot h^{-1}$. The inlet air temperature was set at 160°C and the outlet air temperature was set at 70°C. The homogenized concentrate flow rate was 100 ± 10 $L \cdot h^{-1}$ and the major airflow rate was 2,400 ± 50 kg/h. Fine particles were recycled at the top of the chamber. For T+, the homogenized concentrate received a heat treatment at 75°C for 2 min before spray-drying. For T+++, the homogenized concentrate received a heat treatment at 85°C for 2 min before spray-drying. The whole process for producing T−, T+, and T+++ was duplicated. An average of 100 kg of IF powder was produced for each formula and replicate. Throughout the IF productions, samples were collected in the middle of mixing (**Mix-MID**), after vacuum evaporation (**Mix-EV**), after the addition of oil blend and homogenization (**MixL-HO**), after heat treatment and before spray-drying (only for T+ and T+++; **MixL-HOHT**), and after spray-drying (spray dried powder, **SDP**) at the beginning and at the end of the batch.

Physicochemical Characterization

Dry Matter and Ash Content. The DM and ash contents (g/100 g) were determined in duplicate as described previously (ISO, 1987; Schuck, Dolivet and Jeantet, 2012).

Nitrogen and Protein Contents. Total nitrogen (**TN**) content, non-CN nitrogen content corresponding to the soluble fraction at pH 4.6, and nonprotein nitrogen (**NPN**) content corresponding to the soluble fraction after protein precipitation by trichloroacetic acid 12% (wt/vol) were determined by the Kjeldhal method (IDF, 1993). The protein content was determined as TN subtracted of NPN. The specific N conversion factor for bovine milk protein based IF of 6.38 was used, as reported previously (Maubois and Lorient, 2016). The denaturation extent of WP was calculated by comparison of its measured content (g/kg) in the samples collected throughout the IF production or in the reconstituted IF powders with the theoretical WP content (g/kg); the latter being calculated based on the WP content in the ingredients (MSM and WPI) and their mass used for the IF production.

Individual WP Contents. Quantification of individual WP, α-LA, and β-LG, was conducted after precipitation at pH 4.6 using the reversed-phase HPLC method adapted from Resmini et al. (1989), using a PLRP-S column (gel of polystyrene divinylbenzene, 300 Å, 8 μm, 150 × 3.0 mm) connected to a HPLC System (Dionex UltiMate 3000, Thermo Fisher), equipped with a detector VWD3400RS (Thermo Fisher) operating at 214 nm for the quantification of eluted proteins. Chromatographs were analyzed by Chromeleon software (Version 7.2 SR4, Thermo Fisher). The denaturation extent of individual WP (α-LA or β-LG) was calculated as described above. All measurements were carried out in duplicate.

Total and Free Fat Contents. Total fat content was measured in IF powders by Gerber's acid-butyrometric method (AFNOR, 1990). Free fat content, extracted with petroleum ether, was determined gravimetrically

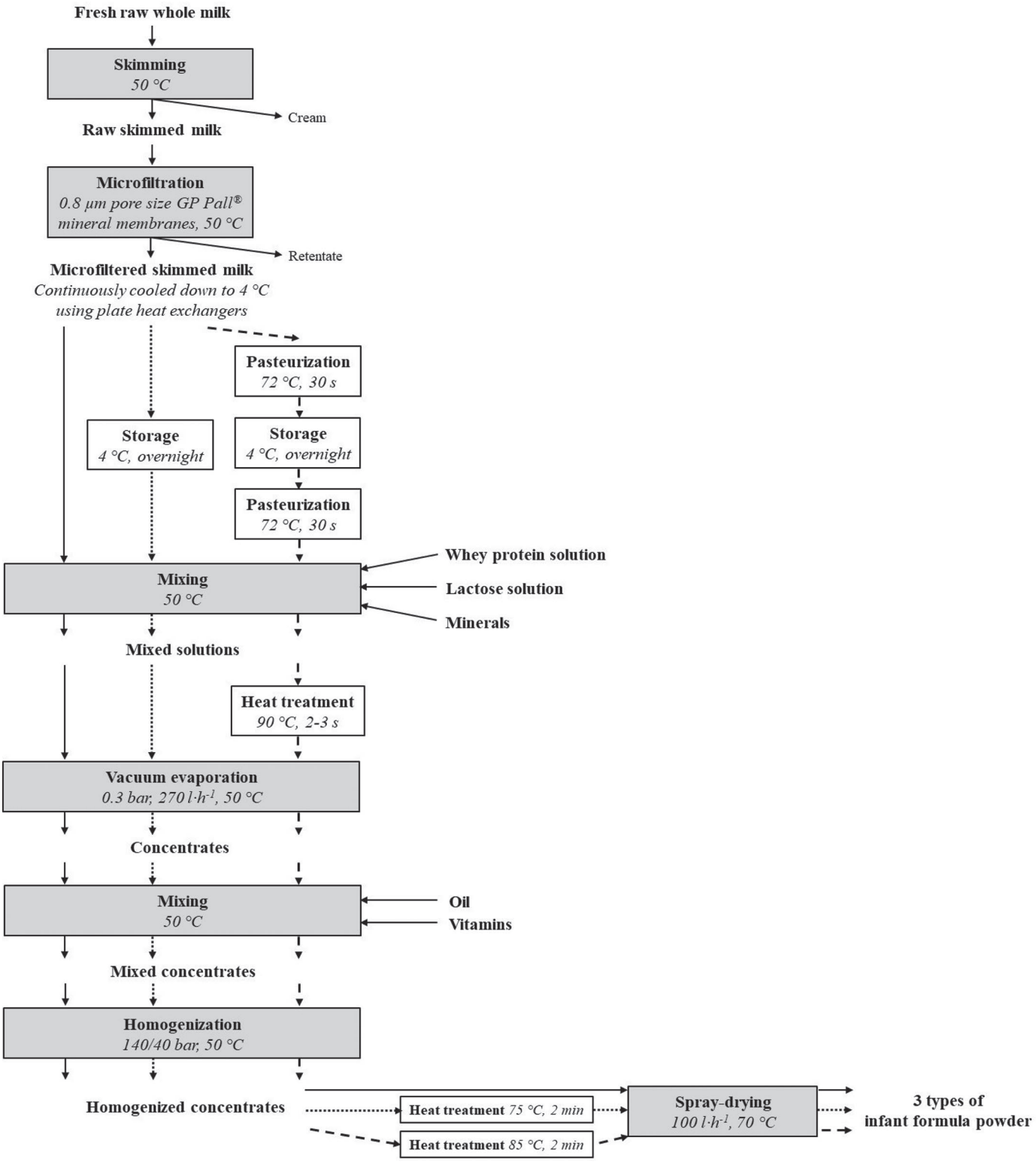

Figure 1. Semi-industrial process flow diagram for production of 3 types of infant formula powders. Solid arrow = T− (no heat treatment), round dot arrow = T+ (moderate heat treatment), hyphen arrow = T+++ (intense heat treatments).

after solvent evaporation. Total and free fat analyses were carried out in duplicate (Schuck et al., 2012).

Mineral Contents. Sodium, potassium, calcium, magnesium, iron, zinc, and copper in IF powders were quantified by atomic absorption spectrometry as described by Brulé et al. (1974). Chloride was quantified with the Sherwood Scientific chloride analyzer (Modele 926, Sherwood Scientific Ltd.) as described by Berdagué et al. (1987). Inorganic phosphorus content was quantified by using the molybdenum blue method according to Jean (1969). All measurements were carried out in duplicate.

Water Activity and Glass Transition Temperature. Water activity (a_w) of IF powders was measured at 25 ± 0.1°C using the Novasina aw-meter (Novasina, Switzerland). The glass transition temperature (**Tg**) was determined using a modulated temperature differential scanning calorimetry method according to Schuck et al. (2012). Water activity and Tg measurements were carried out in triplicate.

Rehydration Properties. Dispersibility and solubility of IF powders were determined according to Schuck et al. (2012). All measurements were carried out in duplicate.

Color Parameters. Infant formula powder color parameters (L = brightness; a* and b* = chromaticity coordinates) were determined in triplicate with a chromameter (Konica Minolta Photo Imaging France SAS). Browning index (**BI**), an indicator of Maillard reaction extent (Martinez-Alvarenga et al., 2014), was calculated using Equations [1] and [2] (Maskan, 2001):

$$BI = \frac{100 \times (x - 0.31)}{0.17}, \quad [1]$$

with

$$x = \frac{a + 1.75 \times L}{5.645 \times L + a - 3.012 \times b}. \quad [2]$$

Particle Size Distribution. Size distribution of the IF powder particles was determined using a laser light-scattering granulometer (Mastersizer, Malvern Instruments Ltd.). The median diameter d(0.5) was chosen to describe the particle size distribution of the IF powders.

Quantification of Maillard Reaction Products

Furosine. The levels of Amadori products (early Maillard reaction products, such as lactulosylysine) in samples collected throughout the IF production and in IF powders were measured after transformation into furosine according to ISO (2004) and Giannetti et al. (2013) with some modifications. Samples adjusted to 3.1% (wt/wt) total protein were hydrolyzed with HCl 8 *M* at 110°C for 23 h under nitrogen. Hydrolyzed samples were purified on a Chromabond 3 mL/500 mg SPE cartridge (Macherey Nagel) using 3 mL of HCl 3 N, after a cartridge conditioning step with 5 mL of methanol and 10 mL of Milli-Q water. The eluate was filtered through a Chromafil xtra 13 mm, 0.2 μm filter and subjected to chromatographic separation. A 25-μL aliquot was injected into the HPLC column (Acclaim Trinity P1, 100 mm × 3 mm i.d., 3-μm pore size) connected to a Dionex UltiMate 3000 HPLC System (Thermo Fisher), equipped with a detector VWD3400RS (Thermo Fisher) operating at 280 nm for the quantification of eluted furosine. The mobile phase composed of a mixture of acetonitrile (phase A), sodium acetate at 0.4% (vol/vol), pH 4.5 (phase B) and KCl 0.27% (wt/vol) in phase B (phase C) was delivered at a flow rate of 0.5 mL/min at 30°C. The gradient started with 60.0% of mobile phase A and 40.0% of mobile phase B and then continued with linear decrease to 10.0% of phase B and increase to 30.0% of phase C in 10 min. This gradient was maintained for 2 min before being brought back to 40% of phase B in 0.5 min. The gradient was then maintained for 5.5 min before the next injection. Furosine in hydrolyzed samples was quantified using a calibration curve established by direct injection of furosine samples at concentration ranging from 1.5 to 18 mg/L. Chromatographs were analyzed by Chromeleon software (Version 7.2 SR4). All measurements were carried out in triplicate after the hydrolysis step.

N^{ε}-Carboxymethyl-Lysine. Sample preparation for CML analysis was adapted from Niquet-Léridon and Tessier (2011). Samples (of different weights, being equivalent to ~10 mg protein) were weighed into 2-mL polypropylene tubes and lyophilized. Their dry weight was then recorded, and the samples reduced by adding 500 μL of 0.1 *M* $NaBH_4$. This reduction step (2.5 h at 20°C) is considered necessary to block the Amadori product and avoid de novo formation of CML during subsequent acid hydrolysis. To each tube, 500 μL of 12 *M* HCl was then added and hydrolysis performed at 110°C for 21 h. Aliquots (200 μL) of hydrolysates were evaporated and resolubilized in 200 μL of 10 m*M* nonafluoropentanoic acid (**NFPA**). All samples were filtered (0.45 μm) before their analysis by tandem MS coupled to liquid chromatography.

Isotope dilution, HPLC with tandem MS detection analyses were performed in multiple reaction monitoring mode on a Waters Quattro Premier XE instrument with a Heated Electrospray Ionization source (HESI–Waters). A Hypercarb column (100 × 2.1 mm, 5 μm; Thermo) with a guard column (10 × 2.1 mm, 5 μm,

same phase) was used for chromatographic separation. An ion-pairing, binary mobile phase flow rate was 0.2 mL/min (A – aqueous 10 m*M* NFPA, B – acetonitrile; percentage of A: 0–9 min, 100–75%; 9–11 min, 75–40%; 11–13 min, 40%; 13.1–21 min, 100%). The following specific transitions were monitored (in elution order): m/z 205.0 → 130.0 and m/z 207.0 → 130.0 for CML and its isotope, respectively. Quantification of all compounds used the ratio of the analyte peak areas or the peak area of its isotope and comparison with 9-point calibration curves. All measurements were carried out in duplicate from the first step of the protocol.

Bacteriological Analysis

Assessment of the bacteriological quality was performed according to the European regulation (Commission of European Communities, 2007), and completed by additional analyses. Numeration of the total aerobic flora at 30°C and of Enterobacteriaceae was conducted throughout the IF production based on AFNOR (2013, 2017a). Numeration of *Enterobacteriaceae*, *Bacillus cereus*, total coliforms, *Cronobacter* spp., *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, total aerobic flora, yeasts and molds in IF powders was conducted by an external accredited laboratory (LABOCEA, Fougères, France) based on AFNOR (2003a,b, 2005, 2009, 2013, 2017a,b,c, 2018).

Statistical Analysis

Statistical analyses were conducted with R, version 3.3.1 (https://www.r-project.org). A nonparametric analysis for repeated measurements was conducted with treatment and production time (and their interaction) as factors, using the nparLD package (Noguchi et al., 2012). For a significant treatment effect ($P < 0.05$), the nparcomp package (Konietschke et al., 2012) was used. For a significant interaction effect, a linear mixed effect model taking into account the repeated measurements was performed and followed by the difflsmeans of the lmerTest package (Kuznetsova et al., 2017). Results are expressed as means ± standard deviation.

RESULTS AND DISCUSSION

Physicochemical Properties of 3 IF Powders

The 3 IF powders, T−, T+ and T+++, complied with the requirements of regulations (European Commission, 2016) in terms of total content of proteins, lipids, lactose, vitamins, and minerals (Table 1 and Supplemental Table S1, https://doi.org/10.15454/DW23PE). The average DM was 97.8 ± 0.1 g/100 g of powder with an average a_w of 0.19 ± 0.01 (Table 2), close to the optimal value of 0.2 as defined by Efstathiou et al. (2002) to ensure the stability of powders during storage. Lipid stability during storage may also influence the nutritional value and flavor of IF powders. The free fat content of the present IF powders was equal to 2.9 ± 0.1% (wt/wt) of total fat content, regardless of the heat treatment intensity. This value is below the critical value (5%) reported by Vignolles et al. (2007) for whole milk powder and should be satisfactory for lipid stability. However, this parameter should be monitored during storage. The storage ability of milk powder also depends very much on its Tg (Schuck et al., 2007). The average Tg of the 3 IF powders (a_w = 0.19) was 61.2 ± 0.4°C. Tham et al. (2017) reported comparable Tg values for IF at a_w of 0.18, which confirmed a good storage ability of IF powders at 25°C, which should be free from physical changes such as stickiness and caking. The median diameter of the powder grains d(0.5) was 170 ± 9 μm, namely in the upper range of nongranulated powders. The 3 IF powders were considered as soluble with a solubility index (**SI**) higher than 99% (Schuck et al., 2012). With a dispersibility index (**DI**) ranging between 97.8 ± 2.0% and 98.9 ± 0.3%, the 3 IF powders could be considered as dispersible. However, it was visually noticeable that T+++ encountered some slight dispersion impairments (flecking) when powder was rehydrated. Concomitantly, a small proportion (2 ± 0.4%) of large particles (25–220 μm) was observed by laser light diffraction solely for this reconstituted formula (data not shown), particles that were dissociated by addition of an anionic dissociating agent (sodium dodecyl sulfate). Thus, these flecks, although present in limited quantity, could be due to some insoluble protein aggregates, either by themselves or acting as bridges between fat droplets through hydrophobic interaction, thus resulting in fat droplet flocculation, such as previously reported (Schmidmeier et al., 2019; Toikkanen et al., 2018).

Bacteriological Quality Throughout the Production

Microfiltration with 0.8-μm pore size membranes has been used as a physical technique to selectively separate microorganisms from the other components of skim milk (i.e., proteins, lactose, minerals) based on size differences. Our choice of using MF membranes with a smaller pore diameter (0.8 μm instead of 1.4 μm commonly used in dairy industry) was based on previous studies reported by Fauquant et al. (2009) and Tomasula et al. (2011), which indicated that 0.8-μm membrane ensured a higher decimal reduction of microflora and both 2 membranes showed a near complete transmission of proteins. The GP concept applied in

Table 1. Average biochemical composition (±SD) of the 3 infant formula (IF) powders over the 2 productions

		Per 100 g of powder			Per 100 kcal			Per 100 kcal	
								UE 2016/127	
Item	Unit	T−	T+	T+++	T−	T+	T+++	Min	Max
Ash	g	3.1 ± 0.6	3.1 ± 0.7	3.3 ± 0.8	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2		
CP[1]	g	11.0 ± 0.8	11.3 ± 0.5	10.7 ± 1.0	2.1 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	1.8	2.5
True protein[2]	g	10.8 ± 0.9	11.1 ± 0.5	10.5 ± 1.0	2.0 ± 0.2	2.1 ± 0.1	2.0 ± 0.2		
CN	%	42 ± 5	41 ± 6	41 ± 2					
WP	%	58 ± 5	59 ± 6	59 ± 2					
Lipids	g	29.6 ± 0.8	30.2 ± 0.1	30.3 ± 0.0	5.6 ± 0.1	5.7 ± 0.0	5.7 ± 0.0	4.4	6.0
Lactose[2]	g	54.5 ± 2.2	53.5 ± 1.1	53.8 ± 1.8	10.3 ± 0.5	10.1 ± 0.1	10.2 ± 0.3	4.5	14.0
Minerals									
Sodium	mg	167 ± 10	161 ± 16	161 ± 15	32 ± 2	30 ± 3	30 ± 3	25	60
Potassium	mg	705 ± 4	663 ± 34	664 ± 17	134 ± 1	125 ± 7	125 ± 4	80	160
Calcium	mg	355 ± 19	373 ± 15	374 ± 13	67 ± 2	70 ± 2	71 ± 2	50	140
Magnesium	mg	47 ± 1	47 ± 0	48 ± 1	9 ± 0	9 ± 0	9 ± 0	5	15
Chloride	mg	518 ± 36	492 ± 10	493 ± 11	98 ± 7	93 ± 1	93 ± 1	60	160
Phosphorus	mg	256 ± 7	245 ± 21	246 ± 33	49 ± 2	46 ± 4	47 ± 6	25	90
Iron	mg	4.1 ± 0.1	4.1 ± 0.2	4.0 ± 0.3	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.3	1.3
Zinc	mg	4.1 ± 0.8	4.1 ± 0.9	4.4 ± 0.8	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.5	1
Copper	µg	440 ± 80	449 ± 99	410 ± 81	83 ± 15	85 ± 19	78 ± 16	60	100

[1]CP content was expressed as required in the UE 2016/127 regulation (i.e., total N × 6.25).

[2]True protein content was determined as total N content subtracted of nonprotein N content, and converted using the specific factor of 6.38.

[3]Lactose content was determined by calculation: Lactose (g/100 g) = DM (g/100 g) − Ash (g/100 g) − Proteins (g/100 g) − Lipids (g/100 g).

our study has several advantages compared with the uniform transmembrane pressure approach, such as low investment and operating costs due to the removal of a permeate recycle pump. As a consequence, no beads were present on the permeate side of the GP membrane, reducing contamination risks for permeate MSM stream. Moreover, GP membranes include a longitudinal permeability gradient built into the support structure without modification of the filtration layer. This design ensures a stable MF operation with uniform permeate flux (J) and transmembrane pressure (Tomasula et al., 2011; Zulewska and Barbano, 2014).

On the other hand, a liquid WPC obtained by MF with 0.1-µm pore size membranes, combined with other filtration techniques, was used instead of cheese whey, a by-product of cheese production, more generally used to re-equilibrate the CN:WP ratio in IF. Separation and purification of WP using membrane filtration preserve WP in their native state and allows to product a more qualitative product. The production of cheese whey involves enzymatic coagulation, acidification, and, most of the time, heat treatment, which may alter the mineral equilibrium and protein integrity, and produce some by-products such as the glycomacropeptide. The latter is a soluble peptide containing 64 amino acid residues, which is released by the chymosin hydrolysis of κ-casein (Britten and Pouliot, 1996). Its presence in cheese whey alters its amino acid profile. Therefore,

Table 2. Physical properties of the 3 infant formula powders over the 2 productions (average ± SD)[1]

Item[2]	T−	T+	T+++
Free fat (% wt/wt total fat)	2.8 ± 0.3	3.0 ± 0.6	2.8 ± 0.3
d(0.5) (µm)	172.12 ± 15.43	177.69 ± 24.82	160.05 ± 6.53
Water activity	0.20 ± 0.02	0.19 ± 0.03	0.19 ± 0.01
DM (g/100 g of powder)	97.9 ± 0.1	97.8 ± 0.0	97.8 ± 0.1
Tg (°C)	61.47 ± 3.44	61.31 ± 7.51	60.74 ± 8.12
Solubility index (%)	>99	>99	>99
Dispersibility index (%)	98.89 ± 0.36	97.77 ± 2.01	98.87 ± 0.25
Color parameters			
L	70.5 ± 0.4	71.0 ± 0.4	72.2 ± 0.0
a*	−3.1 ± 0.5	−3.2 ± 0.7	−3.6 ± 0.7
b*	14.4 ± 0.7	14.3 ± 1.6	14.3 ± 1.3
BI	19.0 ± 0.8	18.5 ± 2.0	17.7 ± 1.4

[1]T−: with no heat treatment; T+: with moderate heat treatment; T+++: with intense heat treatments.

[2]d(0.5) = median diameter of powder grains; Tg = glass transition temperature; L = brightness; a* and b* = chromaticity coordinates; BI = browning index.

for sectors such as IF, which have stringent quality specifications, WPC produced by filtration is an ideal alternative to cheese whey, which also tends toward a global shortage (Henchion et al., 2017).

Over the 2 productions, the MF with 0.8-µm pore size membranes showed its sufficiency in allowing a reduction of bacterial counts of 3 up to 4 $\log_{10}$ for the total aerobic flora, resulting in a microfiltered milk almost free of bacteria (<10 cfu·g^{-1} for an initial population of ~10^4 cfu·g^{-1}) and totally free of *Enterobacteriaceae*. A more specific experiment of MF with 0.8-µm pore size membranes was conducted with nonpathogenic bacteria having morphological characteristics and cell volume close to *Cronobacter spp* (*Lactobacillus casei* and *Propionibacterium freudenreichii*). This confirmed its efficiency to retain these specific bacteria with a reduction of bacterial counts of 4 to 5 $\log_{10}$ (data not shown).

Regarding the WPC obtained from MF 0.1 µm, its bacteriological quality differed between the 2 productions. Although the first production led to a total flora of 1.15 × 10^2 cfu·g^{-1} and an *Enterobacteriaceae* population of 1 cfu·g^{-1}, higher values were obtained for the second production (1.8 × 10^4 and 12 cfu·g^{-1} for total flora and *Enterobacteriaceae,* respectively). This was due to postfiltration contamination, which was more important in the second production.

Throughout the 2 IF productions, both heat treatments applied for T+ (75°C, 2 min on the concentrate) and for T+++ (90°C before evaporation, 85°C for 2 min on the concentrate) allowed a minimal reduction of bacteria counts of 2 $\log_{10.}$ No *Enterobacteriaceae* was detected in the collected samples before spray-drying. The spray-drying step resulted in an average increase of 1 $\log_{10,}$ mainly for T−. The T+ IF, spray-dried after T−, inherited the flora of T−, an effect that decreased along the spray-drying process. This effect was less noticeable for T+++, spray-dried after T+ but with a concentrate sent in the tower at a higher temperature (85°C instead of 75°C).

Overall, the 3 IF powders of the first production presented a satisfactory bacteriological quality with a total flora <10^3 cfu·g^{-1} and an absence of pathogens, including *Salmonella* and *Cronobacter spp* (Table 3). Powder samples collected at the beginning of the spray-drying of T− presented higher but acceptable level of total flora and molds (1.07 × 10^3 and <40 cfu·g^{-1}, respectively) than those analyzed at the end of process (3.63 × 10^2 and <10 cfu·g^{-1}, respectively). This suggests a residual contamination of the spray-drying tower before drying of T−, which was eliminated with the first amount of T−.

During the second production, a total flora of 5.60 × 10^3 and 3.57 × 10^3 cfu·g^{-1} were observed in the powders

Table 3. Bacteriological quality of ingredients and the 3 infant formula powders produced during the first production (T−: with no heat treatment; T+: with a moderate heat treatment; T+++: with intense heat treatments)[1]

Item, cfu/g	Raw skim milk	MSM[2]	Lactose	WP concentrate	T−		T+		T+++	
					Beginning	End	Beginning	End	Beginning	End
Total flora at 30°C	1.32 × 10^4 ±5.18 × 10^3	6	0	1.15 × 10^2 ±2.67	1.07 × 10^3 ±4.00 × 10^2	3.63 × 10^2 ±32.1	4.60 × 10^2 ±28.3	2.20 × 10^2 ±0.00	1.00 × 10^2 ±28.3	95 ±77.8
Bacillus cereus	NA	NA	NA	NA	<10	<10	<10	<10	<10	<10
Total coliforms	NA	NA	NA	NA	<10	<10	<10	<10	<10	<10
Cronobacter spp.	NA	NA	NA	NA	ND/10 g	ND/10 g	ND/10 g	ND/10 g	ND/10 g	ND/10 g
Enterobacteriaceae	1.38 × 10^2 ±1.40	0	0	1	ND/10 g	ND/10 g	ND/10 g	ND/10 g	ND/10 g	ND/10 g
Listeria monocytogenes	NA	NA	NA	NA	abs/25 g	abs/25 g	abs/25 g	abs/25 g	abs/25 g	abs/25 g
Salmonella	NA	NA	NA	NA	abs/25 g	abs/25 g	abs/25 g	abs/25 g	abs/25 g	abs/25 g
Staphylococcus aureus	NA	NA	NA	NA	abs/1 g	abs/1 g	abs/1 g	abs/1 g	abs/1 g	abs/1 g
Yeasts	NA	NA	NA	NA	<10	<10	<10	<10	<10	<10
Molds	NA	NA	NA	NA	<40	<10	<10	<10	<10	<10

[1]NA = not analyzed; ND/10 g = not detected in 10 g; abs/25 g. = absence in 25 g; abs/1 g. = absence in 1 g. MSM = microfiltered skim milk; WP = whey protein.

of T− and T+ with a presence of *Enterobacteriaceae* (73.3 and 10 cfu·g^{-1}, respectively), due to the lower quality of the WP concentrate resulting from post-filtration contamination. The T+ powder could have reached a satisfactory quality if dried independently of T−, but this was not possible with the present experimental design. During this second production, only T+++ showed a satisfactory bacteriological quality with a total flora $<3.00 \times 10^2$ cfu·g^{-1} and an absence of *Enterobacteriaceae*.

Overall, we can conclude that integrating MF of fresh milk into IF production can be efficient to ensure the bacteriological safety of the ingredients without additional heat treatments; however, a high microbiological quality of the ingredients (*Cronobacter*- and *Enterobacteriaceae*-free ingredients); of the processing water, which can be sterilized to ensure its microbiological safety; and of the production environment, by a thorough cleaning, is required. The present experiments were performed at a semi-industrial scale on a workshop pilot not specifically designed for the different successive steps involved, which were thus carried out in an open space. In industry, such production would be performed with a well-adapted continuous processing line, in a controlled environment and with the benefits of continuous improvement process. Such production frame should avoid any postfiltration contamination.

Denaturation Extent of WP

The denaturation extent of WP was followed throughout the production of the 3 types of IF powders by determining the proportion of whey proteins insoluble at pH 4.6, as shown in Figure 2. For T+++, the effect of the double pasteurization (72°C, 30 s) of milk on protein integrity was minimal (denaturation extent <4%). Such HTST pasteurization is widely used in the dairy industry to inactivate pathogenic microorganisms and is proved to cause low levels of denaturation for major WP (α-LA and β-LG) in the milk (Rynne et al., 2004; Rohitha Prasantha and Wimalasiri, 2019; Halabi et al., 2020b). However, HTST can lead to denaturation of some minor proteins, such as lactoferrin, and does not ensure the inactivation of endogenous enzymes, such as plasmin (Rauh et al., 2014; Klotz et al., 2017; Wang et al., 2019). It is noteworthy that, unlike HTST alone, MF allows an efficient removal of somatic cells present in milk containing numerous thermo-resistant enzymes such as plasmin, which can cause proteolysis and thus, off-flavors (Wang et al., 2019).

Regarding vacuum evaporation (0.3 bar, 270 l/h, 50°C) of the mixed solution, this had no effect on the denaturation of WP (from Mix-MID to Mix-EV) in T− and T+. The higher denaturation extent for T+++ in Mix-EV was therefore only due to the heat treatment at 90°C for 2 to 3 s between mixing and evaporation steps; the extent of WP denaturation increased 26 ± 6%. The oil and vitamin addition followed by the homogenization step (140/40 bar, 50°C) had, as expected, virtually no influence on the denaturation of WP (from Mix-EV to MixL-HO). The final heat treatment of the concentrated mix doubled the denaturation extent of WP in both T+ and T+++ (75°C for 2 min and 85°C for 2 min, respectively), although this did not reach statistical significance due to a lack of statistical power. Similar results were reported by previous studies (Singh and Creamer, 1991; Morgan et al., 1999; Mehta and Deeth, 2016) showing that the key factor for protein denaturation during the production of powdered dairy products is the intensity of heat treatments rather than the drying process itself. In addition, it can be noticed that the highest heat treatment (85°C for 2 min applied to the concentrated mix) induced significant whey protein gelation, resulting in pipe clogging at this end of one of the replicates; this never occurred for the production of T− and T+. The denaturation extent of WP tended to decrease for T+++ after spray-drying, although not statistically significant. This can be due to a lack of representativeness of the T+++ sample at

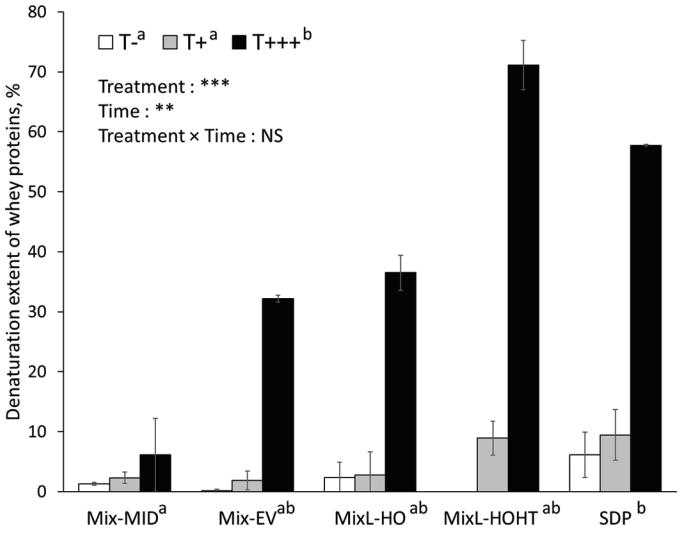

Figure 2. Average denaturation extent (±SD, n = 2) of whey proteins over the 2 productions of the infant formulas powders. T−: no additional heat treatment; T+: moderate heat treatment at 75°C for 2 min, applied before spray-drying; T+++: successive heat treatments at 72°C for 30 s on the milk, 90°C for 2–3 s before evaporation, 85°C for 2 min before spray-drying; Mix-MID: middle point of mixing; Mix-EV: mixture after evaporation; MixL-HO: mixture with lipids after homogenization; MixL-HOHT: mixture with lipids after homogenization and heat treatment (only for T+ and T+++); SDP: spray-dried powder. Statistics were calculated using a nonparametric analysis for repeated measurement. ***$P < 0.001$, **$P < 0.01$, NS: $P \geq 0.05$. Different letters indicate statistically significant differences between treatments or between time ($P < 0.05$).

the MixL-HOHT stage or to some loss of protein aggregates during the process, although this was very limited during spray-drying. The final IF powders produced according to the present processing routes presented a low denaturation extent for T− and T+ (6 ± 4% and 10 ± 4%, respectively) and a high denaturation extent for T+++ (58 ± 0%). The latter value was in accordance with the denaturation extent determined in 4 commercial IF powders (65.0 ± 11.1%). Compared with T− without any heat treatment, T+ might be of particular interest for food safety at the industrial level because the pasteurization step before spray-drying permitted to eliminate pathogens including potential coronaviruses, such as SARS CoV-2 (Duan et al., 2003) or tick-borne encephalitis, which can be present in bovine milk from Central and Eastern Europe and in Asia (Saier et al., 2015), thus further guaranteeing the microbiological quality of the powders without leading to a high protein denaturation extent.

First-age IF is administered to infants as a human milk substitute and thus should mimic this nutritional gold standard as much as possible. The present IF with a low denaturation extent contains mainly native proteins, such as in a raw human milk. Thus, such a minimally processing route can help to develop more biomimetic IF. To get even closer to human milk, the WP profile could also be improved, for instance by the addition of bioactive proteins such as lactoferrin. The present processing would be of particular interest as this protein needs to remain in its native form to preserve its bioactive properties.

Denaturation Extent of Individual WP (α-LA or β-LG)

The denaturation extent of individual WP (α-LA or β-LG) during the production of the 3 types of IF is presented in Figure 3. Heat treatment at 90°C for 2 to 3 s between mixing and evaporation steps induced a loss of native β-LG of 29 ± 10% for T+++, whereas no significant loss of native α-LA was observed. A study by Halabi et al. (2020b) in the context of IF also showed that the decrease of the native WP after heat treatment at 90°C for 15 s was mainly due to the heat denaturation of β-LG. At temperatures above 68°C, β-LG undergoes conformational changes and partially unfolds, exposing its 2 disulfide bridges (Cys66-Cys160 and Cys106-Cys119) and a free sulfhydryl group (-SH group; Cys121). When heated for a short time, the reshuffling of intramolecular disulfide bonds may be responsible for the formation of irreversible non-native monomers of β-LG that can further aggregate (Croguennec et al., 2003; Kleber and Hinrichs, 2007; Wijayanti et al., 2014; Brodkorb et al., 2016; Nielsen et al., 2018), whereas α-LA, without free-SH group, is more heat stable at this point (Mulvihill and Donovan, 1987; Calvo et al., 1993). Heat treatment at 75°C for 2 min had very little influence on the denaturation of α-LA and β-LG for T+, whereas heat treatment at 85°C for 2 min for T+++ induced a denaturation extent of 59 ± 5% for α-LA and 41 ± 8% for β-LG. When heated for a longer time (2 min), although intramolecular reshuffling cannot occur with α-LA because of absence of free -SH group, the intermolecular reshuffling is more active for α-LA than for β-LG because the former possesses more disulfide bonds, leading to higher denaturation extent of α-LA (Wijayanti et al., 2014). As observed for the overall denaturation extent, the denaturation extent of α-LA and β-LG decreased by 26 ± 8% and 14 ± 3% for T+++ after spray-drying.

Effect of Heat Treatment on the Formation of Maillard Reaction Products

The formation of Amadori products, measured as furosine, during the production of the 3 types of IF is presented in Figure 4. The furosine concentration doubled after the addition of vitamins, oil, and pH adjustment

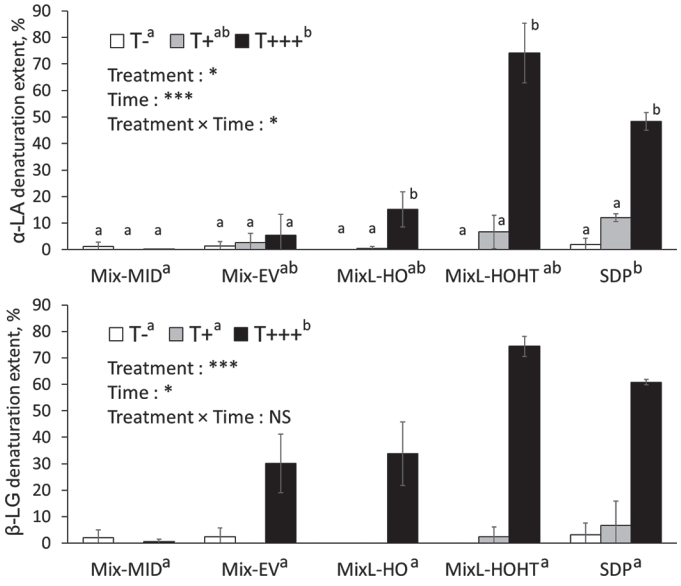

Figure 3. Average denaturation extent (±SD, n = 2) of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) over the 2 productions of the infant formula powders. T−: no additional heat treatment; T+: moderate heat treatment at 75°C for 2 min, applied before spray-drying; T+++: successive heat treatments at 72°C for 30 s on the milk, 90°C for 2–3 s before evaporation, 85°C for 2 min before spray-drying; Mix-MID: middle point of mixing; Mix-EV: mixture after evaporation; MixL-HO: mixture with lipids after homogenization; MixL-HOHT: mixture with lipids after homogenization and heat treatment (only for T+ and T+++); SDP: spray-dried powder. Statistics were calculated using a nonparametric analysis for repeated measurement. ***$P < 0.001$, **$P < 0.01$, NS: $P \geq 0.05$. Different letters indicate statistically significant differences between treatments or between time ($P < 0.05$).

followed by homogenization (140/40 bar, 50°C) for all the 3 IF. Vitamin C can act as precursor or intermediate for the formation of Maillard reaction products (Pischetsrieder et al., 2005; Troise et al., 2016). After encapsulation of vitamin C, an increase of 45% of available lysine and a decrease of 10 to 53% in furosine and CML concentrations were observed in milk (Troise et al., 2016). In addition, pH adjustment of the mix from acidic pH after the addition of vitamins to more neutral values (pH from 6.02 ± 0.08 to 6.6 ± 0.2) after addition of potassium hydroxide can also accelerate the initial step of the Maillard reaction by increasing the nucleophilicity of protein amino groups (Lund and Ray, 2017; Nunes et al., 2019). Heat treatment at 75°C for 2 min for T+ and 85°C/2 min for T+++ made their furosine concentration doubled again from 255 ± 18 to 445 ± 208 mg/100 g MAT and from 249 ± 36 to 582 ± 170 mg/100 g MAT. In contrast, the 3 IF powders reached similar furosine concentrations after spray-drying, in accordance with previous studies, in which Henle et al. (1995) reported furosine concentration from 930 to 1,890 mg/100 g of proteins in commercial IF powders determined by isocratic ion-pair reversed-phase HPLC method. Lower values have been reported by Birlouez-Aragon et al. (2004) using ion-pair HPLC, ranging from 170 to 370 mg/100 g of proteins in 17 commercial IF powders, possibly due to a higher rate of transformation of the Amadori products into advanced products of the Maillard reaction.

Concomitantly to the evolution of furosine, the CML concentration doubled after the addition of vitamins, oil, pH adjustment followed by homogenization, and after heat treatments of the concentrate before spray-drying for T+ and T+++ (Figure 4). After spray-drying, the CML concentration was numerically lower in powder T− than in powder T+++ (8.8 ± 0.2 and 10.6 ± 0.0 mg/100 g MAT, respectively), but this did not reach statistical significance due to the variability observed along the entire production and due to the limited number of production replicate (n = 2). Contrary to the WP denaturation extent, which was more reproducible, a high variability was observed regarding furosine and CML between the 2 IF productions, particularly from the step of vitamin and oil addition, pH adjustment, and homogenization until spray-drying. The Maillard reaction products are likely to be more affected by some slight difference of the production parameters (pH, temperature) inherent to the semi-industrial level. Nevertheless, the present values are in the range of those previously reported by Delatour et al. (2009), who analyzed the CML concentrations in 7 commercial IF powders using the same technique as in the present study and indicated an average value of 7.6 mg/100 g MAT.

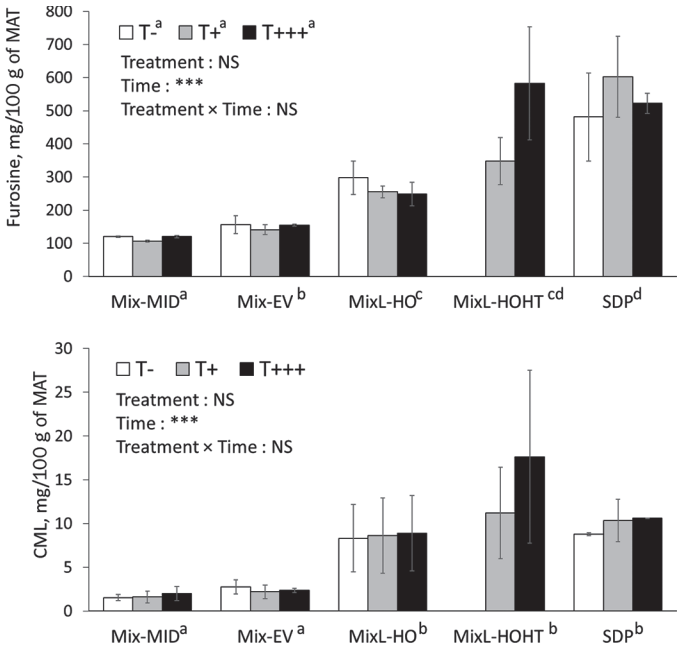

Figure 4. Average furosine (as a surrogate marker of the Amadori products) and Nε-carboxymethyl-lysine (CML) concentration (± SD, n = 2) over the 2 productions of the infant formula powders. T−: no additional heat treatment; T+: moderate heat treatment at 75°C for 2 min, applied before spray-drying; T+++: successive heat treatments at 72°C for 30 s on the milk, 90°C for 2–3 s before evaporation, 85°C for 2 min before spray-drying; Mix-MID: middle point of mixing; Mix-EV: mixture after evaporation; MixL-HO: mixture with lipids after homogenization; MixL-HOHT: mixture with lipids after homogenization and heat treatment (only for T+ and T+++); SDP: spray-dried powder. Statistics were calculated using a nonparametric analysis for repeated measurement. ***$P < 0.001$, **$P < 0.01$, NS: $P \geq 0.05$. Different letters indicate statistically significant differences between treatments or between time ($P < 0.05$).

The concentration of the Maillard reaction products of the 3 IF powders was in accordance with their BI, one of the first indicators of the extent of the Maillard reaction. The latter was in the same range among the 3 IF powders, varying from 17.7 up to 19 (Table 2). This value was related to a pale yellow color, which was induced not only by the Maillard products but also by the yellow-color vitamin mix.

IF powders are products devoted to be stored for several months, during which the Maillard reaction products may increase. To this end, these compounds will be followed during the storage step and will be presented in a future study.

The present study presents some limitations. First, due to the scale of the production, only 2 replicates could be performed, which limits the statistical power. The present study has demonstrated that a native IF powder could be bacteriologically safe, however this does not guarantee viral decontamination, which, if suspected, could be performed by the moderate heat

treatment, depending on the virus. Further validation is required concomitantly to the development of viral analyses in dairy products. Finally, the viscosity of the product along IF processing could not be followed, however no problem of processability was encountered except for T+++, such as stated above.

CONCLUSIONS

This study demonstrated the feasibility of producing, at semi-industrial scale, a bacteriologically safe IF powder with a high content of native proteins, based on membrane filtration of fresh milk, low temperature unit operations, and high level of bacteriological quality of the ingredients. Adding a moderate heat treatment step (75°C, 2 min) on the concentrate before spray-drying could further guarantee the microbiological quality of the IF powder, if dried independently, without leading to a high protein denaturation extent. The absence of heat treatment on the IF tended to limit the formation of advanced Maillard reaction products (CML); however, the extent of the difference was much lower on these products than on the WP denaturation extent. This study opens up new avenues for the production of minimally processed IF powders, although the nutritional and physiological impacts of such IF remain unknown. Such minimally processing route can be highly relevant for IF including heat-sensitive bioactive components, such as lactoferrin.

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