Effect of processing on polyamine content and bioactive peptides released after in vitro gastrointestinal digestion of infant formulas

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ABSTRACT

This study examined the influence of processing on polyamines and peptide release after the digestion of a commercial infant formula designed for children during the first months of life. Polyamine oxidase activity was not suppressed during the manufacturing process, which implicates that polyamine concentrations were reduced over time and during infant formula self-life. In gel electrophoresis, in vitro gastrointestinal digestion of samples with reduced amount of enzymes and time of digestion shows an increase in protein digestibility, reflected in the increase in nonprotein nitrogen after digestion and the disappearance of β -lactoglobulin and α -lactalbumin bands in gel electrophoresis. Depending on the sample, between 22 and 87 peptides were identified after gastrointestinal digestion. A peptide from β -casein f(98–105) with the sequence VKEAMAPK and antioxidant activity appeared in all of the samples. Other peptides with antioxidant, immunomodulatory, and antimicrobial activities were frequently found, which could have an effect on infant health. The present study confirms that the infant formula manufacturing process determines the polyamine content and peptidic profile after digestion of the infant formula. Because compositional dissimilarity between human milk and infant formula in polyamines and proteins could be responsible for some of the differences in health reported between breast-fed and formula-fed children, these changes must be taken into consideration because they may have a great effect on infant nutrition and development.

Key words: infant formula, polyamine, peptide, simulated gastrointestinal digestion, mass spectrometry

INTRODUCTION

Breast milk has a complex composition of nutrients and bioactive components designed to fulfill the needs of the growing infant. In recent years, the infant food industry has made an effort to develop infant formulas that are more similar to human milk to improve the nutrition of infants who are not breastfeeding.

Protective compounds, such as cytokines, oligosaccharides, and even microbes, in breast milk provide the newborn with the means to adapt to the environment (Gueimonde et al., 2007; Newburg and Walker, 2007). Among the bioactive compounds found in breast milk are polyamines, such as, spermidine, spermine, and putrescine, as well as bioactive peptides released during milk protein digestion. Polyamines have a positive effect on the development of the gastrointestinal tract (Larqué et al., 2007) and immune system (Pérez-Cano et al., 2010; Gómez-Gallego et al., 2014b). The levels and effects of these compounds in infant formulas compared with human milk are of special interest, because their concentrations are lower than in human milk (Buts et al., 1995). Moreover, dietary proteins are a source of biologically active peptides that are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. Once bioactive peptides are liberated, they may act as regulatory compounds. Bioactive peptides are widely distributed among milk protein sequences (Clare and Swaisgood, 2000), which can be released during digestion in vivo. However, changes that take place in protein structure during the manufacturing of infant formulas can influence protein digestion and peptide liberation (Korhonen et al., 1998).

Technological processes used in food manufacturing affect the functional, nutritional, and biological properties of food components. Depending on the intensity of the heat treatment, the nutritive value of proteins can be affected in a positive or negative way (Korhonen et al., 1998) and, by extension, can affect other related compounds.

The structure of milk is greatly altered depending on the various mechanical and thermal steps of the

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processing chain (Michalski and Januel, 2006). Heating and homogenization are the most common and most widely used methods capable of modifying proteins during infant formula manufacturing. Among the different physical and chemical changes, a great deal of attention has been focused on the covalent interaction of protein-carbohydrate via the Maillard reaction. During this reaction, the conjugation of a reducing carbohydrate to the 3-amino group of lysine occurs spontaneously under heating conditions (Corzo-Martínez et al., 2012). Some studies have shown contradicting effects regarding the Maillard reaction. On one hand, glycation can lead structural changes, which could generate new enzymatic cleavage sites (Corzo-Martínez et al., 2012; Joubran et al., 2015) modulating protein digestibility and peptides release after digestion. On the other hand, conjugation could limit enzymatic accessibility through steric hindrance (Joubran et al., 2015). These 2 counteracting effects could explain the different digestive patterns reported for other authors. Moreover, heat treatment changes the effect of homogenization on milk structure (Michalski and Januel, 2006). The main effect of homogenization on soluble milk components is the disruption of casein micelles in micellar form or as fragments. Homogenization seems to improve milk digestibility. However, heat treatment changes the effect of homogenization on milk structure, and it has

been reported that infants better digest native human milk fat globules than homogenized droplets from infant formula (Michalski and Januel, 2006). As concluded by Michalski and Januel (2006), the structural consequences in milk proteins seem to depend on the sequence of the homogenization and heat treatments, but they are rather controversial because of the various treatments applied and to the different procedures used in the food industry.

The aims of this study were to evaluate how formula processing influences polyamine content and peptide release after digestion. Furthermore, the behavior of the polyamine oxidase activity of the milk and the digestibility of proteins were studied. The results of this work could be a preliminary step to improving infant formula composition, which could promote better health status of children fed with infant formulas during the first months of life.

MATERIALS AND METHODS

Samples

The infant formula samples used in this study were supplied by Hero España S.A. (Alcantarilla, Spain) at different representative stages along the manufacturing process. Figure 1 shows the flow diagram of the infant

Figure 1. Diagram of infant formula manufacturing process. Samples were taken in the steps labelled with a black circle, from originally raw milk (F1) to final infant formula ready for sale (F5). In the first concentration stage, the product was concentrated by 30 to 40%. The process included a two-step homogenization. In the fluidized bed spray-dryer tower the water was removed to a final moisture of 3%. F1 = cow milk; F2 = cow milk after skimming and the first thermal treatment; F3 = concentrated milk after the second thermal treatment; F4 = concentrated infant formula after the last thermal treatment; F5 = the infant formula final product; and SW = milk whey used as an ingredient.

formula manufacturing process and the steps at which the samples were taken. The samples were (**F1**) cow milk used as raw material in infant formula processing; (**F2**) cow milk after skimming and the first thermal treatment; (**F3**) concentrated milk after the second thermal treatment; (**F4**) concentrated infant formula after the last thermal treatment; (**F5**) the infant formula final product; and (**SW**) milk whey used as an ingredient. Whey was demineralized sweet whey from cow milk, with a content of lactose around 70%, added as an ingredient to increase milk serum proteins in the final product to 60% of total proteins.

One kilogram of powdered infant formula (F5) and 500-mL aliquots of liquid samples (F1, F2, F3, F4, and SW) were taken from 5 different batches separately. Liquid samples were lyophilized, and all of the samples were preserved at -20°C until analysis.

Determination of Moisture and Nitrogen

Moisture (method 964.22) along the infant formula manufacturing process and nitrogen (N) content using the micro-Kjeldahl procedure (method 955.04) were determined in the samples and digestions using official AOAC methods (AOAC, 1990). Protein calculations were made using 6.25 as the conversion factor. Nonprotein nitrogen in the samples and digestions was estimated using the micro-Kjeldahl method after dissolving 20 g of the sample in 100 mL of 15% TCA for milk protein precipitation and filtration.

Analysis of Polyamines

An HPLC method using a diode array detector was used. The HPLC system consisted of a Waters 2690 system connected to a Waters 910 detector. The analytical column was a Spherisorb 5.0- μm ODS2, 4.6 mm $\times$ 150 mm (Waters Corp., Milford, MA). Detection was performed at 254 nm.

The samples were diluted 10 times with a solution of TCA as described by Nishibori et al. (2007), but the concentration was adjusted to 15% for milk protein precipitation. The samples were homogenized using gentle agitation for 30 min (Pollack et al., 1992). After centrifugation at $13,000 \times g$ for 15 min at 4°C , the supernatants were filtered using 0.45- μm membrane filters (Whatman, Brentford, UK) and dansylated by adapting the method described by Buts et al. (1995). Clear supernatant (1 mL) was basified by adding 250 μL of saturated sodium carbonate and 1 mL of dansyl chloride solution (10 mg/mL of acetone; Fluka, Steinheim, Germany).

After incubation, 200 μL of L-proline solution (Scharlau, Barcelona, Spain) was added to clean the excess

Table 1. Gradient of acetonitrile:water used to separate dansylated polyamines

Time (min)	Acetonitrile (%)	Water (%)
0	60	40
5	60	40
15	70	30
20	95	5
25	98	2
26	98	2
26.1	61	39
30	60	40

dansyl chloride (Escribano, 1990). Extraction of dansyl derivatives was performed twice with cyclohexane, and organic phases were collected and evaporated in nitrogen air flow. Residues of dansyl derivatives were dissolved in 1 mL of acetonitrile (Merck, Darmstadt, Germany) and filtered with 0.45- μm HPLC filters (Upchurch Scientific, Oak Harbor, WA). Aliquots of 20 μL were injected in the HPLC. Samples were run for 30 min according to a linear gradient method including 2 mobile phases: water and acetonitrile (Table 1).

Each sample was analyzed in duplicate. Quantification of the polyamine concentration was made by comparing the integrated surface areas of peaks with areas of dansylated polyamines of known concentration on a standard curve. To avoid mistakes due to loss of dansylated polyamines during manipulation, 1,3-diaminopropane (Aldrich, Steinheim, Germany) was used as the internal standard (Tovar, 2002). Polyamine concentrations were expressed in milligrams per kilogram.

Analysis of Polyamine Oxidase Activity

The polyamine oxidase (PAO) activity assay was performed using the method described by Suzuki et al. (1984) in which hydrogen peroxide formed by polyamine oxidase is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound by peroxidase, adding pargyline and semicarbazide for monoamine oxidase and diamine oxidase inhibition, respectively. A multimode microplate reader (Synergy 2; Bio-Tek, Winooski, VT); fluorescence filters with excitation and emission wavelengths of 360 and 460 nm, respectively; and 96-well black, flat-bottomed microplates were used to carry out this assay. Each sample was analyzed in triplicate. Results were expressed as micromoles of H_2O_2 per milligram of dry extract per 30 min.

In Vitro Gastrointestinal Digestion of Samples

The hydrolysis procedure was based on the method described by Miller et al. (1981) and comprised the simulation of gastric and intestinal digestion of the samples

by *in vitro* enzymatic treatment. The modifications introduced by different authors (Bosscher et al., 2001; Jovani et al., 2001; Hernández-Ledesma et al., 2007; Frontela et al., 2008; Bourlieu et al., 2014) reduced the concentrations of digestive enzymes and the time of digestion, to simulate gastrointestinal conditions during lactation. In the first step, 3.9 g of the dry extract of the sample was dissolved in 10 mL of Milli-Q water obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Then, the pH was adjusted to 4.0 with 1 M HCl, and the samples were hydrolyzed with pepsin (from porcine gastric mucosa; Sigma, St. Louis, MO) at a ratio of 10.7 mg of pepsin per gram of sample, for 30 min at 37°C. Directly afterward, the pH of the digested sample was raised to 5.0 using sodium hydrogen carbonate 1 M, and the samples were hydrolyzed with pancreatin (from porcine pancreas; Sigma) and bile salts (Fluka), at a ratio of 2 mg of pancreatin per gram of the sample and 6.25 g of bile salts per gram of pancreatin. Intestinal digestion with pancreatin was performed at 37°C for 90 min. Digestion was carried out in a thermally controlled incubator under constant stirring. The reaction was stopped, the sample was put on ice, and the pH was adjusted to 7.2 with sodium hydrogen carbonate 1 M. The enzymes were inactivated by heating at 95°C for 15 min in a water bath, followed by cooling to room temperature. A fraction of the digestions were run in gel electrophoresis to check for the presence of proteins and changes in protein composition. Digestibility and reverse-phase-HPLC-tandem mass spectrometry analyses were performed to evaluate influence of the processing in peptide release after digestion. *In vitro* gastric and intestinal digestions and subsequent analyses were carried out at least in duplicate.

Protein digestibility was calculated as the increase in NPN following gastrointestinal digestion, as described by Rudloff and Lönnerdal (1992). Nitrogen content of fractions was determined as described previously, employing the Kjeldahl procedure.

Gel Electrophoresis

To investigate the protein composition of milk fractions during digestion, the molecular weights of the proteins were analyzed by gel electrophoresis in SDS. The analysis was conducted using a PhastSystem electrophoresis system (Pharmacia, Uppsala, Sweden) as described by Jiménez-Saiz et al. (2011), with precast homogenous gels 20% (GE Healthcare, New York, NY) and PhastGel SDS buffer strips (Amersham Biosciences Corp., Uppsala, Sweden), following the manufacturer's instructions for the electrophoretic and Coomassie staining conditions. The samples were dissolved in 10-

mM Tris-HCl buffer, pH 8.0, containing 2.5% SDS and 10 mM EDTA, and heated at 95°C. They were then analyzed in the presence of 5% 2-β-mercaptoethanol.

Analysis by Online Reverse-Phase-HPLC-Tandem Mass Spectroscopy

A water-soluble hydrolysate extract was obtained by centrifugation at $20,000 \times g$ for 30 min at 5°C and by filtration through a Whatman no. 41 filter (GE Healthcare Bio-Sciences, Pittsburgh, PA). The water-soluble extract was subjected to ultrafiltration through a hydrophilic 1,000-Da cutoff membrane (Amicon Inc., Beverly, MA).

The hydrolysates were injected into an HPLC system (Agilent, Santa Clara, CA), which was connected online to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik, Bremen, Germany), according to the method of Hernández-Ledesma et al. (2004). The column used in these experiments was a 250-mm $\times$ 4.6-mm Discovery BIO Wide Pore C18 column (Bio-Rad, Richmond, CA). A Nova-Pak C18 guard column 20 mm $\times$ 3.9 $\times$ 4 μ m (Waters Corp.) was used to protect the analytical column. The injection volume was 50 μ L. Solvent A was a mixture of water and trifluoroacetic acid (1,000:0.37, vol/vol), and solvent B contained acetonitrile and trifluoroacetic acid (1,000:0.27, vol/vol). Peptides were eluted with a linear gradient of 0 to 45% solvent B over 60 min at a flow rate of 0.8 mL/min. The signal threshold to perform auto mass spectrometry(n) analyses was 1,000, and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35 to 1.4 V. The m/z spectral data were processed using Data Analysis 3.0 (Bruker Daltonik) and transformed to spectra representing mass values. Mass spectrometry(n) spectra were processed in BioTools 2.1 (Bruker Daltonik) to perform peptide sequencing.

Statistical Analysis

Statistical analyses were conducted using ANOVA and relevant post hoc tests with SPSS 15.0 software (IBM Corp., Armonk, NY). Differences between means were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of Processing on Polyamine Content and Polyamine Oxidase Activity of Infant Formulas

The concentrations of the polyamines detected in the analyzed samples varied over a wide range, probably reflecting differences in the stages of the process from

Table 2. Polyamine concentration (mg/kg) and range in dry samples along manufacturing process (mean with range in parentheses)

Sample ¹	Putrescine	Spermidine	Spermine
F1	0.650 ^a (0.576–0.749)	0.057 ^a (0–0.124)	0.065 ^a (0–0.140)
F2	1.047 ^{ab} (0.680–1.380)	0.126 ^a (0.116–0.151)	0.125 ^{ab} (0.124–0.129)
F3	1.858 ^{abc} (0.170–3.950)	0.967 ^{bc} (0.038–1.779)	0.469 ^{abc} (0–1.084)
F4	2.824 ^c (1.369–7.757)	1.118 ^c (0.661–2.009)	0.756 ^{bc} (0.376–1.367)
F5	2.441 ^{bc} (1.339–3.492)	0.576 ^{ab} (0.271–0.983)	0.466 ^{abc} (0.137–1.210)
SW	3.109 ^c (1.670–5.610)	0.406 ^a (0–0.761)	0.948 ^c (0–1.935)

^{a-c}Different letters in the same column indicate statistical significant differences at the level of $P < 0.001$.

¹F1 = cow milk; F2 = cow milk after skimming and the first thermal treatment; F3 = concentrated milk after the second thermal treatment; F4 = concentrated infant formula after the last thermal treatment; F5 = the infant formula final product; and SW = milk whey used as an ingredient.

where the samples were taken, the original raw milk of the batch, and the PAO activity of the sample. The results are summarized in Table 2. Overall, polyamine content was lower in the first steps of the processing than in the last steps or final product. This could be due to the PAO activity in these samples (Table 3) influenced for whey addition before the last concentration stage. With respect to PAO activity of the raw milk, the final infant formula retained more than 60% of the enzymatic activity, which was not inactivated because of the high resistance of the enzyme. This persistent PAO activity changes polyamine concentrations over time and during infant formula reconstitution.

As reported in previous studies (Pollack et al., 1992; Romain et al., 1992; Buts et al., 1995), the polyamine content in infant formula is around 10 times less than that of human milk. In our samples, if the formula was reconstituted according to the manufacturer's instructions, the final concentrations of putrescine, spermidine, and spermine would be 0.317, 0.075, and 0.061 mg/kg, respectively. These amounts were similar to those reported by Romain et al. (1992), with higher levels

for putrescine. Previous studies (Pollack et al., 1992; Romain et al., 1992; Buts et al., 1995; Dorhout et al., 1996) established a great variation in milk composition from one mother to another and even between the left and right breasts (Buts et al., 1995), but the average concentrations of polyamines were around 0.058, 0.580, and 0.825 mg/kg for putrescine, spermidine, and spermine, respectively. Thus, the concentrations of spermidine and spermine, the most active polyamines (Larqué et al., 2007) in manufactured formulas, were much lower than in human milk, although the concentration of putrescine was higher than in breast milk. This finding reveals the lack of an important functional compound in infant formulas compared with breast milk, and because of the role of polyamines on immune-system and intestinal-microbiota development, we recently reported (Gómez-Gallego et al., 2014a,b) that the development of infant formulas with a high but safe polyamine content must be taken into consideration.

Effect of Processing on Digestibility of Milk Proteins in Infant Formulas

The protein composition of fractions was further characterized by gel electrophoresis. We found that the processing of infant formulas had an influence on the protein structure. The comparison between raw milk (F1) and infant formula (F5) shows that processing alters bands on SDS gels (Figure 2; lanes 1 and 2, versus 5 and 6). As reported by other authors (Barbé et al., 2013), heat treatment induced susceptibility to hydrolysis, thereby facilitating protein digestion, which was reflected in band comparison after gastric and gastrointestinal digestion (Figure 2; lanes 3 and 4, versus 7 and 8). It is mainly evident in the β -LG and α -LA bands, which persist in raw milk after gastrointestinal digestion and practically disappear during digestion in infant formula samples. In raw milk, β -LG and α -LA remain undegraded because of the partial resistance to pepsin and pancreatin in the soft acidic conditions of

Table 3. Polyamine oxidase (PAO) activity (μ mol of H_2O_2 /mg of dry extract per 30 min) in samples along manufacturing process and evolution of the percentage of relative PAO activity comparing with raw milk

Sample ¹	PAO activity (mean $\pm$ SD)	Relative PAO activity (%)
F1	21.59 ^a $\pm$ 2.31	100.00
F2	25.27 ^a $\pm$ 0.72	117.04
F3	11.94 ^b $\pm$ 1.43	55.30
F4	11.98 ^b $\pm$ 3.58	55.49
F5	14.48 ^b $\pm$ 0.45	69.38
SW	15.44 ^b $\pm$ 3.23	71.51

^{a,b}Different letters in the same column indicate statistical significant differences at the level of $P < 0.001$.

¹F1 = cow milk; F2 = cow milk after skimming and the first thermal treatment; F3 = concentrated milk after the second thermal treatment; F4 = concentrated infant formula after the last thermal treatment; F5 = the infant formula final product; and SW = milk whey used as an ingredient.

infant *in vitro* digestion (Korhonen et al., 1998). In the whey samples, proteins seemed to have relatively high resistance to the conditions of digestion, which was reflected in the persistence of the bands for β -LG and α -LA, even after *in vitro* digestion (Figure 2; lane 12). These results are in accord with data published with other authors, demonstrating that milk proteins persisted longer under infant digestion conditions (Joubran et al., 2015), potentially generating bioactive peptides.

Digestibility was related to the increase in NPN that resulted from enzymatic digestion, as shown in Table 4. Protein digestibility, related to the increase in NPN, was lower in whey (SW), which supported the results confirmed by gel electrophoresis. It appeared that the manufacturing process exerted whey protein denaturation, which facilitated its digestion. At the end of the F5 sample digestion, estimated *in vitro* digestibility of the F5 sample increased from 17.9% of the F1 sample to 44.9%. As reported by Rudloff and Lönnerdal (1992), this low digestibility could be due to the soft gastrointestinal digestion conditions during the first months of life as well as lipid-protein or peptide interactions. But, our results were even lower than those reported by other authors.

It is important to consider that *in vitro* digestibility is only an approximation of the physiological process and does not take into account other characteristics of real digestion, such as gastric emptying, intestinal fluid, intestinal motility, and mucosal enzymes. However, it could be considered a warning regarding real protein digestion during lactation (Binaghi et al., 2002).

Identified Peptides After Gastrointestinal Digestion

The ultrafiltration permeates of the digested samples were obtained and subjected to tandem mass spectrometry for peptide identification. A total of 49 protein fragments could be identified for raw milk (F1; Supplemental Table S1), 29 for skim milk (F2; Supplemental Table S2), 22 for concentrated milk (F3; Supplemental Table S3), 23 for milk serum (SW; Supplemental Table S4), 55 for concentrated manufactured formula (F4; Supplemental Table S5), and 87 for infant formula (F5; Supplemental Table S6). All supplemental files are at <http://dx.doi.org/10.3168/jds.2015-10030>.

Evidence is growing that the peptide sequences released during digestion can vary depending on the manufacturing process of infant formulas. As suggested by other authors (Chang et al., 1981), digestion experi-

Figure 2. Sodium dodecyl sulfate-PAGE of raw milk (F1); infant formula (F5); and whey (SW) proteins and their fractions along gastrointestinal digestion: lane 1 = raw milk; lane 2 = raw milk at infant gastric pH (pH = 4); lane 3 = raw milk after infant gastric digestion; lane 4 = water-soluble extract of raw milk after infant gastrointestinal digestion (at pH = 7); lane W = molecular weight marker (97 kDa = phosphorylase b from rabbit muscle; 60 kDa = BSA; 45 kDa, ovalbumin; 30 kDa = carbonic anhydrase from bovine erythrocytes; 20.1 kDa = soybean trypsin inhibitor; 14.4 kDa = bovine alpha lactalbumin); lane 5 = infant formula reconstituted per manufacturer's indications; lane 6 = infant formula at infant gastric pH (pH = 4); lane 7 = infant formula after infant gastric digestion; lane 8 = water-soluble extract of infant formula after infant gastrointestinal digestion (at pH = 7); lane 9 = milk serum; lane 10 = serum at infant gastric pH (pH = 4); lane 11 = serum after infant gastric digestion; lane 12 = water-soluble extract of milk serum after infant gastrointestinal digestion (at pH = 7). The positions of lactoferrin (a), serum albumin (b), α_{s2} -CN (c), α_{s1} -CN (d), β -CN (e), κ -CN (f), β -LG (g), and α -LA (h) are indicated.

ments with infant formulas may provide information regarding the susceptibility of proteins to gastrointestinal conditions, hydrolysis, and bioactive peptide release in infant formulas.

The comparison of the majority of peptide profiles in the 1,000-Da permeates showed that changes in the profile of peptides released and identified was greatly dependent on the processing stage of the infant formula. Our results showed that the percentage increases of peptides originating from whey proteins during processing—from 33.3% in raw milk (F1) to 49.9% in final infant formula (F5)—may have been due to the addition of whey (SW) as an ingredient.

With the exception of the whey samples (SW), a peptide from β -CN f(98–105) with the sequence VKEAMAPK and antioxidant activity (Korhonen and Pihlanto-Leppäla, 2007) appeared in all of the samples. Other abundant peptides in the samples were ETYG, ELLK, STAV, LPDT, AVVKK, ATAST, IIAEK, EAMAPK, RELEE, RYLG, LRLK, LRLKK, LKKI, VQVTSTAV, YLGY, YSLA, YLGYL, VEGPKLV, and HAQ. Among these peptides, the α_{s1} -CN fragment f(91–94), with the sequence YLGY, had a structure potentially able to act as an opioid receptor ligand. This peptide is a fragment of α -casomorphin (Clare and Swaisgood, 2000), which maintains an amino-terminal tyrosine for essential opioid activity (Chang et al., 1981) and an aromatic AA in the third or fourth position that favors peptide union to opioid receptor (Kohmura et al., 1990). Further studies are necessary to confirm the potential opioid activity of this fragment, because it may have an important role in sleeping pattern and the development and function of the gastrointestinal tract in infants depending on its opioid agonistic or antagonistic activity (Wada and Lönnerdal, 2014).

Additionally, some of the peptide sequences found in F1, F2, and F5 samples have been found to exert

angiotensin I-converting enzyme (ACE)-inhibitory activities. Their sequences were also described in human milk proteins, and it can be expected that breast-fed infants as well as infants fed with milk-based formulas will obtain ACE-inhibitory peptides, which may play a role in cardiovascular health. It has been proposed that the immaturity-related higher serum ACE activity in early life could program cardiovascular disease later in life but that ACE-inhibitory peptides present in milk might improve this negative effect (Wada and Lönnerdal, 2014). So their presence in formulas and their activity compared with breast milk should be an object of further studies.

Moreover, in the profile of peptides released after gastrointestinal digestion of the final manufactured formula (Supplemental Table S6; <http://dx.doi.org/10.3168/jds.2015-10030>), peptides with antioxidant (HIQKEDVPSE, VKEAMAPK; Korhonen and Pihlanto-Leppäla, 2007; Gupta et al., 2010), antimicrobial activity (IPAVFK; Pellegrini et al., 2001), ACE-inhibitory peptides (FRQF, PLW, RPK, RVY, IIAEK; Maruyama et al., 1987; Matsufuji et al., 1994; Nagaoka et al., 2001; Shahidi and Zhong, 2008; Contreras et al., 2009), a peptide modulator of nonclassical cadherins functions (QUINNK; Blaschuk et al., 1999), and a cathepsin B inhibitor (PFPGP; Lee and Lee, 2000) were found. It is important to highlight that our results shows some fragments originating from β -LG, a protein absent in human milk. Two of these peptides—RVY and IPAVFK—were reported to exert ACE-inhibitory and antibacterial activity, respectively. Additional studies are necessary to determine whether these peptides have similar functional properties to human milk, defined for a different peptide profile (Wada and Lönnerdal, 2014). In addition, the fragments of many previously characterized bioactive peptides were found after infant formula (F5) gastrointestinal digestion. Some of these

Table 4. Digestibility of proteins in samples along infant formula manufacturing process

Sample ¹	Moisture (%)	Protein in dry extract (mg/g)	NPN before digestion in dry extract ² (mg/g)	NPN after digestion in dry extract ² (mg/g)	Digestibility ² (%)
F1	88.17	26.84	0.50 ± 0.21	1.27 ± 0.21	17.89 ± 5.2 ^b
F2	91.18	37.45	0.35 ± 0.11	1.23 ± 0.26	14.87 ± 4.64 ^b
F3	60.38	10.89	0.18 ± 0.12	0.97 ± 0.20	45.64 ± 11.80 ^a
F4	61.11	11.23	0.24 ± 0.03	1.03 ± 0.21	44.99 ± 11.57 ^a
F5	1.69	10.57	0.19 ± 0.02	0.95 ± 0.20	45.19 ± 11.29 ^a
SW	79.14	69.72	0.90 ± 0.09	1.45 ± 0.08	4.90 ± 1.29 ^c

^{a-c}Different letters in the same column indicate statistical significant differences at the level of $P < 0.001$.

¹F1 = cow milk; F2 = cow milk after skimming and the first thermal treatment; F3 = concentrated milk after the second thermal treatment; F4 = concentrated infant formula after the last thermal treatment; F5 = the infant formula final product; and SW = milk whey used as an ingredient.

²Data of NPN and digestibility represent mean ± SD.

fragments may have preserved similar bioactivity of the origin sequence and must be taken in consideration for further studies.

The differences observed in the peptidic profile in each step of the processing could be related to the sequence and conditions of heat treatments along the manufacturing process. Because Maillard conjugation could modulate the functionality and digestibility of milk proteins, the knowledge of how processing under well-controlled conditions changes the peptides release after gastrointestinal digestion could be useful for improving functionality through the generation of bioactive peptides (Corzo-Martínez et al., 2012; Joubran et al., 2015).

Antimicrobial and immunomodulatory peptides, together with polyamines, may be responsible for the different susceptibility to some diseases between formula-fed and breast-fed infants. Angiotensin I-converting enzyme-inhibitory peptides may play a role in cardiovascular health (Wada and Lönnerdal, 2014), and antioxidant peptides may protect infants from oxidative stress-associated diseases, such as necrotizing enterocolitis (Okur et al., 1995).

CONCLUSIONS

The present study confirms that the infant formula manufacturing process determines, together with raw milk composition, the polyamine content and peptidic profile after digestion of the infant formula. Therefore, compositional dissimilarity between human milk and infant formula in polyamines and proteins, and the functionality of these proteins and their peptides, could be responsible for some of the differences in health reported between breast-fed and formula-fed children. These changes must be taken into consideration because they may have a great effect on infant nutrition and development. Further studies are important to address the effects of the manufacturing process in protein digestibility, peptides profile, and polyamine content during formulation of infant formulas for the potential implication in human health.

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