# Bacteriological, biochemical and immunological properties of colostrum and milk from mothers of extremely preterm infants

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## ABSTRACT

**Objectives:** The objective of this work was to elucidate the influence of extremely premature birth (gestational age: 24-27 weeks) on the microbiological, biochemical and immunological composition of colostrum and milk.

**Materials and Methods:** A total of 17 colostrum and 34 milk samples were provided by the 22 mothers of extreme that participated in this study. Bacterial diversity was assessed by culture-based methods while the concentration of lactose, glucose and *myo*-inositol was determined by a gas chromatography procedure. Finally, the concentrations of a wide spectrum of cytokines, chemokines, growth factors and immunoglobulins were measured using a multiplex system.

**Results:** Bacteria were present in a small percentage (<35%) of the colostrum and milk samples. Staphylococci, streptococci and lactobacilli were the main bacterial groups isolated from colostrum and they could be also isolated, together with enterococci and enterobacteria, from some milk samples. The colostrum concentrations of lactose and glucose were significantly lower than those found in milk while the contrary was observed in relation to *myo*-inositol. The concentrations of most cytokines and immunoglobulins in colostrum were higher than in milk, and the differences were statistically significant for $IgG_3$, $IgG_4$, IL-6, INF-γ, IL-4, IL-13, IL-17, MCP-1 and MIP-$1_\beta$.

**Conclusions:** The bacteriological, biochemical and immunological content of colostrum and milk seems to be specifically adapted to the gestational age. Efforts have to be done in order to try that preterm neonates receive milk from their own mothers or from donors matching, as much as possible, the gestational age of the preterm.



**Key Words:** preterm, colostrum, milk, bacteria, glucose, lactose, *myo*-inositol, cytokine, immunoglobulin

## INTRODUCTION

Breastfeeding is the natural and best advisable way of supporting the growth and development of healthy term infants (Agostini et al., 2009). The benefits of breast milk are well recognized as providing health benefits in early infancy and extending into adulthood (Eidelman et al., 2012). In addition, current research confirms that breast milk with appropriate fortification is the optimal care for the low and the very low birth weight infant (Wight et al., 2008; Schanler, 2007; Lee et al., 2012). When breastfeeding may not be possible and own mother's milk may not be available, donor human milk becomes the next alternative (American Academy of Pediatrics, 2012; Arslanoglu et al., 2013),

The benefits of breast milk for preterm infants include faster gastric emptying (Ewer and Yu, 1996; Schanler et al., 1999b); a faster tolerance to enteral feeding and a reduced need of parental nutrition (Schanler, 2000), enhanced nutrient absorption (Hamosh, 1994); improved visual and cognitive development (Hart et al., 2003; Schanler et al., 2005; DiBiasie, 2006; Sacker et al., 2006), and reduced incidence of necrotizing enterocolitis (NEC), sepsis, and other infections (Lucas and Cole, 1990; Pisacane et al., 1992; Rønnestad et al., 2005; Sisk et al., 2007; Chauhan et al., 2008). Such effects are probably due to the combined action of nutrients and a variety of bioactive factors present in colostrum and breast milk, such as inmunoglobulins, inmunocompetent cells, antimicrobial fatty acids, polyamines, oligosaccahrides, lysozyme, lactoferrin, and other glycoproteins, antimicrobial peptides, *myo*-inositol and, also, commensal or potentially probiotic bacteria (Ballard and Morrow, 2013; Fernández et al., 2013).

It is long known that the concentration of many, if not all, nutrients and bioactive compounds changes from colostrum to mature milk and, in addition, there is a variability associated to several factors, such as host' genetic background, health status, nutrition, lactation stage (infant's age), circadian rhythm, milk fraction (foremilk, hindmilk), geographic location, etc. (Ballard and Morrow, 2013).

Gestational age may also influence the concentration of nutrients and bioactive compounds (Montagne et al., 1999; Dvorak et al., 2003; Koenig et al., 2005; Moltó-Puigmartí et al., 2011; Zhang et al., 2013). Biochemical and immunologic data suggest that mothers' colostrum or milk feedings may provide the greatest protection from infection for the most immature infants (Rodriguez et al., 2009) which, in comparison to larger preterm infants, are the most immunocompromised, are exposed routinely to invasive, lifesaving procedures and remain in the pathogen-laden neonatal intensive care unit (NICU) for the longest period of time. However, studies on the composition of colostrum and milk from mothers delivering before the 30th week of gestation are scarce and only the macronutrient milk composition was studied (McLeod et al., 2013).

In this context, the objective of this work was to study a wide variety of microbiological, biochemical and immunological parameters in milk of mothers of very preterm infants (<27th week of gestation), and to evaluate differences between colostrum and milk from these mothers.

## MATERIAL AND METHODS

### Colostrum and milk samples

Colostrum (n=17) and milk (n=34) samples (~2 ml) were obtained from 22 mothers of very preterm infant who gave birth at the Hospital Universitario La Paz

(Madrid, Spain) (Table 1). Sampling was performed following a specific protocol approved by the local ethical committee and informed consent was obtained from each donor. Samples were aliquoted and stored at -20ºC until analysis.

**Bacterial cultures and identification of isolates**

Adequate peptone water dilutions of the samples were plated onto Man, Rogosa and Sharpe (MRS; Oxoid, Basingstoke, UK) supplemented with L-cysteine (0.5 g/L) (MRS-C; a medium for isolation of lactic acid bacteria), TOS-Propionate (TOS; Merck, New Jersey, USA; a medium for isolation of bifidobacteria), MacConkey (MCK; BioMérieux, Marcy l'Etoile, France; a medium for isolation of enterobacteria) and Columbia Nadilixic Acid (CNA, BioMérieux; a highly nutritious, general-purpose medium for the isolation and cultivation of fastidious microorganisms) agar plates. MCK and CNA plates were aerobically incubated at 37ºC for 24 hours while MRS-C and TOS plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an anaerobic workstation (MINI-MACS, DW Scientific, Shipley, UK) at 37ºC for 48 h.

After incubation and counting, one representative of each colony morphology type was selected from each plate. These isolates were grown in BHI or MRS-cys broth and stored at -80°C in the presence of glycerol (30%, v/v). All the isolates were identified by MALDI-TOF mass spectrometry in a Vitek-MS™ instrument (BioMérieux, Marcy l'Etoile, France) in the facilities of Probisearch (Tres Cantos, Spain).

**Analysis of lactose, glucose and *myo*-inositol**

The concentrations of lactose, glucose and *myo*-inositol were determined by gas chromatography with flame ionization detector (GC-FID) in 17 colostrum and 34 milk samples following the method described by Montilla et al. (2005), with minor modifications. For this purpose, 0.2 mL of sample was made up to 2 mL with methanol in a volumetric flask to remove proteins and fat. Mixtures were gently stirred, followed by standing for at least 1 h at room temperature until the supernatant became transparent. The clear supernatant was employed for carbohydrate analysis and ~~a solution of 0.04% (~~ phenyl-β-D-glucoside (0.04% w/v in ethanol/water 70:30, v/v) was used ~~added~~ as internal standard.

Before derivatization, equal volumes (0.5 mL) of supernatant and internal standard solution were mixed and dried at 38 to 40ºC in a rotary evaporator. For derivatization 150 µL of *N*-trimethylsilylimidazole were added to silylate the carbohydrates, and the reaction was completed in 30 min at 70ºC. Silylated carbohydrates were extracted with 0.3 mL of hexane and 0.4 mL of water. Volumes in the range of 1 to 2 µL of the organic phase containing silyl derivatives were injected into the column.

The trimethylsilyl ethers of carbohydrates were analysed in an Agilent Technologies 7890A gas chromatograph equipped with a commercial 30 m × 0.32 mm inside diameter, 0.5 µm film fused silica capillary column SPB$^{TM}$–17, bonded, crosslinked phase (50% diphenyl/50% dimethylsiloxane) (Supelco, Bellefonte, PA, USA). Separation was performed at 235ºC for 9 min, followed by an increase up to 270ºC at rate of 15ºC/min and maintenance of this temperature for 15 min. Temperatures of the injector and flame ionization detector were 280 and 290ºC, respectively. Injections were carried out in split mode 1:30, using 1 mL/min of nitrogen as carrier gas. Data acquisition and integration were performed using Agilent Chem-

146 Station Rev. B.03.01 software (Wilmington, DE). To calculate ~~study~~ the response factor
147 relative to the internal standard, five standard solutions containing lactose, glucose and
148 *myo*-inositol were prepared over the expected concentration range in milk and
149 colostrum samples (1 to 8 g/L for lactose and from 5 to 200 mg/L for *myo*-inositol and
150 glucose). Response factors were calculated after triplicate analysis of standard solutions.
151 The identity of carbohydrates present in colostrum and milk samples was confirmed by
152 comparison with relative retention times of corresponding standards ~~solutions~~.

153 **Immunological analysis**

154 The concentration of 18 cytokines, chemokines and growth factors, including
155 interleukin (IL) IL-1β, IL-6, IL-12(p70), interferon-γ (INF-γ), tumor necrosis factor-α
156 (TNF-α), IL-2, IL-4, IL-10, IL-13, IL-17, IL-8, growth related oncogene-α (GRO-α),
157 macrophage-monocyte chemoattractant protein (MCP-1), macrophage inflammatory
158 protein-1β (MIP-1β), IL-5, IL-7, granulocyte colony stimulating factor (G-CSF) and
159 granulocyte-macrophage colony stimulating factor (GM-CSF), was determined in 16
160 colostrum and 22 milk samples using a Bioplex 200 system instrument (Bio-Rad,
161 Hercules, CA) and the Bio-Plex Pro™ Human Cytokine, Chemokine and Growth Factor
162 Assays (Bio-Rad). Previously, and to avoid interferences in the immunoassay, the fatty
163 layer and the somatic cells were removed from the samples. Briefly, sample aliquots (1
164 ml) were centrifugated at 800 × *g* for 15 min at 4ºC and the intermediate aqueous phase
165 was collected and stored at -20ºC until analysis. All the determinations were carried out
166 by duplicate following the manufacturer's protocol, and standard curves were
167 performed for each analyte.

168 The concentration of immunoglobulin (Ig) IgG1, IgG2, IgG3, IgG4, IgM and
169 IgA was determined in the same samples using the Bio-Plex Pro™ Human Isotyping
170 Assay kit (Bio-Rad) in the Bioplex 200 system instrument. For this purpose, the

171 samples were conditioned as described above for cytokine analysis. Analyses were
172 carried out by duplicate following the manufacturer's protocol and standard curves were
173 performed for each analyte.
174
175 **Statistical analysis**
176 Microbiological data, recorded as colony forming units (CFU) per mL, were
177 transformed to logarithmic values before statistical analysis. Biochemical data (sugars
178 and bioactive compounds) were tested for normality of distribution by Shapiro-Wilk
179 tests. Normal data were expressed as the mean and 95% confidence interval (CI) of the
180 mean, while not normal data were expressed as the median and interquartile range
181 (IQR).
182 The contingency tables of the detection frequencies of bacterial species,
183 cytokines and immunoglobulins were obtained, and then differences were evaluated
184 with the Fisher test; when required the Yates correction was applied. Relations between
185 epidemiological information were evaluated with the Fisher test; when required the
186 Yates correction was applied. Differences in mayor bacteria species counts, Shannon-
187 Weaver diversity index (SDI) and sugar concentrations in colostrum and mature milk
188 were evaluated using one-way ANOVA when the variable was normally distributed,
189 and the Kruskal-Wallis test when not. Levels of immunoglobulins and cytokines in the
190 11 paired samples were compared with the paired t-test when the variable was normally
191 distributed and with the Wilcoxon signed-rank test when not. Correlation analysis of all
192 the quantitative variables measured was carried out; Pearson correlation coefficient and
193 statistical signification were calculated. Carbohydrate levels, from all the samples, and
194 immunocompound levels, from the colostrum samples, and the possible relation with
195 the age of lactation were evaluated by correlation analysis. Differences were considered

196 significant at $P < 0.05$. Statgraphics Centurion XVI version 16.1.15 (Statpoint
197 Technologies Inc., Virginia, USA) software was used to perform these analyses.
198
199 **RESULTS**
200
201 **Study participants**
202 A total of 22 women participated in this study and provided 19 colostrum and 34
203 milk samples. The characteristics (age, week of pregnancy, way of delivery,
204 single/twins pregnancy, antibiotic and corticosteroid treatment, chorioamnionitis) of
205 study participants are presented in Table 1. All of them gave birth between weeks 24th
206 and 27th (14% at week 24th, 18% at week 25th, 36% at week 26th and 32% at week
207 $27^{th}$). Some relationships were observed when frequencies of these characteristics'
208 categorical factors were cross-tabulated and an independency test was carried out (Table
209 2). Week of birth was related to mother's age ($P = 0.045$), being younger those women
210 who delivered at the shortest gestational ages; similarly, a diagnosis of chorioamnionitis
211 was more frequent as the gestational age was shorter ($P = 0.012$), and as the mother's
212 age was lower ($P = 0.038$). Finally, antibiotic treatment was related with C-section
213 deliveries ($P = 0.000$).
214
215 **Bacterial counts and identification of the isolates**
216 Viability of bacteria using both general and selective growth media was
217 evaluated in the 17 colostrum and 34 milk samples. Low detection frequencies were
218 observed in both types of samples since bacteria could only be isolated from a low
219 percentage of them (Table 3). When bacterial growth was detected, the bacterial counts
220 oscillated between 2.00 and 3.28 $\log_{10}$ CFU/mL and between 2.00 and 4.19 $\log_{10}$

221 CFU/mL in colostrum and milk samples, respectively (Table 3). The main genera
222 isolated from colostrum were *Staphylococcus*, *Streptococcus* and *Lactobacillus* while,
223 in addition to these genera, Enterococcus and enterobacteria were also among the main
224 microorganisms isolated from milk (Table 3). The detection frequencies of enterococci
225 ($P$ = 0.000), lactobacilli ($P$ = 0.041) and enterobacteria ($P$ = 0.038) in milk samples
226 were statistically higher than in the colostrum ones (Table 3). The mean for
227 staphylococci counts was higher in colostrum while those for streptococci and
228 lactobacilli were higher in milk although such differences did not reach a statistically
229 significant level. In relation to the Shannon-Weaver diversity index, no significant
230 differences were observed between the colostrum and the milk samples analyzed in this
231 study.
232
233 **Analysis of lactose, glucose and *myo*-inositol**
234 The mean, minimum and maximum values of lactose, *myo*-inositol and glucose
235 concentrations found in the colostrum and milk samples are shown in Fig. 1. Mean
236 (95% CI) concentration values of lactose were 56.9 (52.1-61.7) and 62.5 (60.4-64.7) g/L
237 in the colostrum and the milk samples, respectively. *Myo*-inositol and glucose were also
238 detected and quantified; *myo*-inositol concentrations were 300.6 (249.7-351.5) mg/L
239 and 194.27 (172.5-216.0) mg/L in the colostrum and the milk samples, respectively,
240 while those of glucose were 109.3 (44.2-154.4) and 588.8 (152.8-845.1), respectively.
241 Although the levels of these three compounds showed a certain degree of
242 variability depending on the women, the mean concentrations of colostrum's lactose,
243 *myo*-inositol and glucose were statistically different from those found in the milk
244 samples (Fig. 1); glucose ($P$ = 0.000) and lactose ($P$ = 0.013) concentrations were

245 significantly higher in mature milk while that of *myo*-inositol ($P$ = 0.000) was
246 significantly higher in the colostrum ones.
247
248 **Immunological analysis**
249 The concentration of a variety of cytokines, chemokines, growth factors and
250 immunoglobulins in 16 colostrum and 11 mature milk (milk obtained ≥ 21 days after
251 birth) samples were measured in this study (Table 4). Globally, the values obtained for
252 all these immune factors showed a high degree of variability depending on the donor, a
253 fact that is reflected in the CI or IQR values obtained for some of the analyzed
254 parameters, such as $IgG_1$, IgM, IgA, IL-$1_\beta$, IL-6, INF-γ, IL-17, IL-8, IL-7 and G-CSF.
255 All the immunoglobulins (with the exception of IgG2 detected in 43% of the
256 samples), together with IL-$1_\beta$, IL-6, IL-8, GRO-α, MCP-1, MIP-1β, IL-7 and G-CSF,
257 could be detected in all the colostrum samples. In contrast, GM-CSF (1 sample), IL-2 (3
258 samples) and IL-10 (5 samples) showed the lowest detection frequencies in these
259 samples (Table 4).
260 In relation to mature milk, all the immunoglobulins were also detected in all the
261 samples with the exception, again, of $IgG_2$, which could not be detected in any sample.
262 IL-8, MCP-1 and MIP-1β could also be detected in all the samples while in the case of
263 IL-$1_\beta$, IL-6, GRO-α, IL-7 and G-CSF, there was only a single sample in which they
264 could not be detected (Table 4). In addition to $IgG_2$, INF-γ, IL-2, IL-4 and IL-17 were
265 not detected in any milk sample (Table 4).
266 The mean concentration of all the immune compounds in the colostrum samples
267 was higher than in the milk ones, with the exceptions of IL-13 and GRO-α (Table 4).
268 Despite the high degree of variability depending on the donor, statistically significant
269 differences between colostrum and milk samples were found for the concentrations of

$IgG_3$ ($P$ = 0.017), $IgG_4$ ($P$ = 0.031), IL-6 ($P$ = 0.036), INF-γ ($P$ = 0.000), IL-4 ($P$ = 0.000), IL-13 ($P$ = 0.000), IL-17 ($P$ = 0.000), MCP-1 ($P$ = 0.006) and MIP-$1_\beta$ ($P$ = 0.000) (Table 4). Statistically significant differences were also observed in relation to the detection frequencies of $IgG_2$ ($P$ = 0.045), IL-12(p70) ($P$ = 0.000), INF-γ ($P$ = 0.005), TNF-α ($P$ = 0.012), IL-4 ($P$ = 0.000), IL-13 ($P$ = 0.001), IL-17 ($P$ = 0.000), IL-5 ($P$ = 0.003) and GM-CSF ($P$ = 0.001), being all of them more frequently detected in colostrum than in milk samples (Table 4).

## DISCUSSION

Staphylococci, streptococci and lactobacilli were the main bacterial groups isolated from colostrum and they could be also isolated, together with enterococci and enterobacteria, from some milk samples. In the last decade, breast milk has been recognized as a source of commensal and potential probiotic bacteria, including the bacterial groups cited above (Fernández et al., 2013). The bacterial concentration (2-4 log cfu/ml) was similar to the values previously reported from colostrum and milk of healthy women (Perez et al., 2007; Jiménez et al., 2008; Gómez de Segura et al., 2012). In contrast, bacteria could be detected only in a small percentage (<35%) of the colostrum and milk samples analyzed in this study while previous studies have shown that milk of most (if not all) women contain detectable levels of viable bacteria (Heikkela and Saris, 2003; Martín et al., 2003; Marín et al., 2009; Joost et al., 2013).

The low bacterial detection frequency observed among colostrum and milk of extreme preterm babies mothers may be due to two reasons. First, it has been pointed that a specific mammary microbiota is formed during late pregnancy through a entero-mammary mechanism involving gut monocytes (Jeurink et al., 2013); this process,

295 involving a physiological bacterial translocation from the gut to mesenteric lymph
296 nodes and mammary gland, seems to be exacerbated in the last weks prior to term
297 delivery (Perez et al., 2007). Therefore, bacterial colonization of the mammary glands
298 of extreme preterms' mothers may be, at least, minimum and it may account for the
299 lower detection frequency found in this study. Some culture-dependent and -
300 independent studies have confirmed a vertical mother-to-infant bacterial transfer of
301 maternal gut bacteria via breast milk (Albesharat et al., 2011; Makino et al., 2011;
302 Martín et al., 2012; Jost et al., 2014). In addition, two studies that focused on the oral
303 administration of three lactobacilli strains isolated from human milk provided new
304 evidences that show the existence of a bacterial entero-mammary pathway during
305 lactation (Jiménez et al., 2008; Arroyo et al., 2010).

306 The second reason for a low bacterial detection frequency is the high percentage
307 of women that received antibiotherapy among those that participated in this study.
308 Recently, it has been reported that the number of lactobacilli- or bifidobacteria-positive
309 milk samples was significantly lower in women that had received antibiotherapy during
310 pregnancy or lactation (Soto et al., 2014). It is well known that antibiotics are
311 responsible for dysbiosis processes in the human microbiota and, it is becoming evident
312 that antibiotherapy during pregnancy, intrapartum and during lactation alters the
313 maternal microbiota (Murk et al., 2011).

314 The transition from colostrum to mature breast milk during early puerperium
315 is associated with significant concentration changes of numerous compounds,
316 including concentrations of free sugars and polyols. It has been repeatedly observed
317 that, after the first days of a term postpartum, the concentrations of lactose and
318 glucose increase significantly (Neville et al., 1984; van Beusekom et al., 1993,
319 Coppa et al., 1993; Cavalli et al., 2006; Gabrielli et al., 2011; Jóźwik et al., 2013).

The results of this work showed that the glucose and lactose concentrations in the examined population had also a significant upward trend from colostrum to milk. Although previous studies reported that preterm milk generally has lower lactose levels than term milk (Coppa et al., 1997; Cavalli et al., 2006), the colostrum and milk concentrations of lactose observed in this study were similar to those obtained with the same methodology in mothers of term neonates (Gómez de Segura et al., 2012; Espinosa-Martos et al., 2013). With respect ~~to outliers~~ of glucose content, five samples had a concentration greater than 1 g/L, these data could be due to that the mothers may suffer diabetes. Whitmore et al. (2012) observed that milk from diabetic mothers had a glucose content three times higher than milks of mothers without any health problem. This fact could be consider normal taking into account that glucose transport into milk is dependent upon blood glucose concentrations (Neville et al. 1990). On the other hand, three samples had lactose content less than 40 g/L, probably because these mothers may suffer mastitis. Neville et al. (1984) found low levels of lactose and high levels of sodium and chloride in milks from women with mastitis.

In contrast to lactose or glucose, the concentrations of *myo*-inositol decrease significantly over the first 4 days of lactation (Pereira et al., 1990; van Beusekom et al., 1993; Espinosa-Martos et al., 2013; Jóźwik et al., 2013), a fact that has been observed in this study, too. *Myo*-inositol promotes maturation of several components of surfactant and may play a critical role in fetal and early neonatal life (Howlett et al., 2012). In a pioneer study, Bromberger and Hallman (1986) found striking differences in the *myo*-inositol concentration of infant feedings (preterm colostrum, term colostrum; mature milk; infant formulas; parenteral nutrition). More specifcically, colostrum from mothers

345 who delivered prematurely had the highest *myo*-inositol concentration and was
346 significantly higher that colostrum from mothers who delivered at term. *Myo*-inositol
347 concentrations in colostrum were significantly higher than those in mature milk while
348 preterm and mature milk *myo*-inositol levels did not differ significantly from each other.
349 The results obtained in our work confirm such findings since the *myo*-inositol
350 concentration in colostrum samples (mean: 300.6 mg/L) was significantly higher than in
351 the mature milk ones (mean: 194.3 mg/L). In addition, the colostrum concentration
352 found in this study (from samples provided by mothers of extreme preterms) was
353 notably higher than that reported in a previous work (Espinosa-Martos et al., 2013)
354 when colostrum samples from mothers of term babies were analyzed using the same
355 procedure and analytical technique (mean: 243.3 mg/L). The administration of *myo*-
356 inositol to premature infants with respiratory distress syndrome who are receiving
357 parenteral nutrition during the first week of life is associated with increased survival
358 without bronchopulmonary dysplasia and with a decreased incidence of retinopathy of
359 prematurity (Hallman et al., 1986). Recently, a Cochrane revision AÑO ??? concluded
360 that *myo*-inositol supplementation results in statistically significant and clinically
361 important reductions in important short-term adverse neonatal outcomes, and that A
362 multicenter randomized controlled trial of appropriate size is justified to confirm such
363 findings (Howlett et al., 2012). Therefore, the results of this study highlight the
364 importance of own's mother colostrum for preterm neonates.

365 In agreement with our findings, previous studies have reported that the amounts
366 of several cytokines, chemokines, growth factors and immunoglobulins, with potential
367 anti-infectious, anti-inflammatory and immunomodulatory roles, are notably higher in
368 colostrum than in milk (Islam et al., 2006, Buescher, 2001, Garofalo, 2010).
369 Interestingly, the colostrum of mothers of very preterm infants analysed in this study

showed higher concentrations of relevant immunocompounds, such as IgA, IL-6, TNF-α, IL-4, IL-17, MCP-1, MIP-1β, IL-5, IL-7 or G-CSF, than those observed in term colostrum samples using the same procedure (Espinosa-Martos et al., 2013). It has been previously reported that, globally, immunoprotection provided by colostrum and milk increases as the gestational age (and, as a consequence, the neonate maturity) decreases (Montagne et al., 1999, Araujo et al., 2005, Koeing et al., 2005, Castellote et al., 2011). Maturation of the mammary gland seems to parallel that of the infant and, as a result, the closure of the tight junctions in the mammary epithelium may be delayed following preterm birth. This suggests that the immune components that are unique to colostrum may be especially protective during the first week of life and, particularly, to very preterm infants, a population at the highest risk for feeding intolerance and nosocomial infection (Weterbeek et al., 2006, Thompson & Bizzarro, 2008). Therefore, it would be highly desirable to start, as soon as possible, trophic feeds using own mother colostrum in very preterm infants since it is easy and inexpensive procedure and it is and well-tolerated by even the smallest and sickest very preterm infants (Rodriguez et al., 2010).

In conclusion, a better knowledge on the composition of preterm colostrum and milk will help neonatologists and human milk banks to improve and optimize existing feeding strategies and to design novel alternatives.

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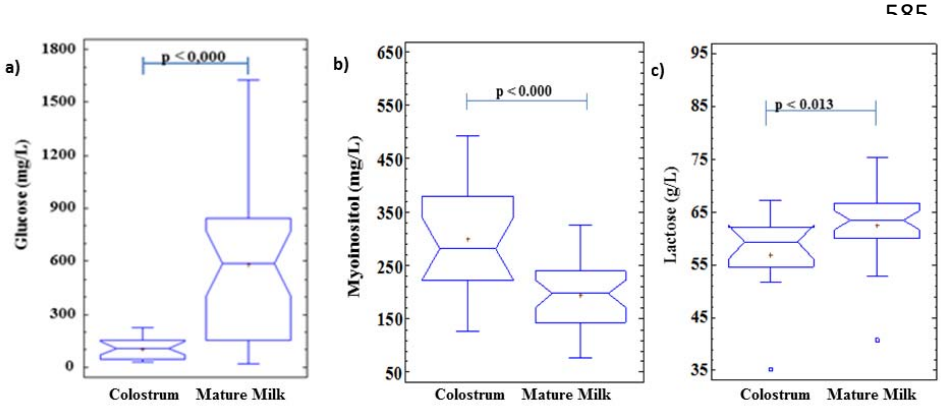
579

## Legend to figures

**FIGURE 1.** Concentration of glucose (a), *myo*-inositol (b) and lactose (c) in colostrum (N=17) and mature milk (N=34) samples from mothers of very preterm infants. Kruskal-Wallis test was used for glucose, which values were not normally distributed while one-way ANOVA was used for *myo*-inositol and lactose.



593 **Table 1:** Characteristics of study participants (n=22).

| Characteristic | Number (%) |
|---|---|
| **1. Mother's age** | |
| < 28 | 6 (27) |
| 28-35 | 7 (32) |
| >35 | 9 (41) |
| **2. Gestational age (weeks)** | |
| 24 | 3 (14) |
| 25 | 4 (18) |
| 26 | 8 (36) |
| 27 | 7 (32) |
| **3. Type of delivery** | |
| Vaginal | 8 (36) |
| C-section | 14 (64) |
| **4. Singleton/twins** | |
| Single | 18 (82) |
| Twins | 4 (18) |
| **5. Use of antibiotics** | |
| No | 7 (32) |
| Yes | 15 (68) |
| **6. Use of corticosteroids** | |
| No | 6 (27) |
| One dose | 5 (23) |
| Two doses | 11 (50) |
| **7. Chorioamnionitis** | |
| No | 14 (64) |
| Yes | 8 (36) |

594

595 **Table 2:** Potential correlations among the characteristics of study participants (n=22).

| p-value | Gestational age | Type of delivery | Antibiotic | Corticosteroids | Chorioamnionitis | Mother's age* |
|---|---|---|---|---|---|---|
| **Gestational age** | | 0.095 | 0.179 | 0.438 | 0.012 | 0.045 |
| **Type of delivery** | | | 0.000 | 0.181 | 0.326 | 0.513 |
| **Antibiotic** | | | | 0.071 | 0.673 | 0.244 |
| **Corticosteroids** | | | | | 0.334 | 0.053 |
| **Chorioamnionitis** | | | | | | 0.038 |
| **Mother's age*** | | | | | | |

596 * Mother's age was categorized into tree levels according to data distribution: under 25 percentile (<28), between
597 25-75 percentiles (28-35) and over 75 percentile (>35).
598

599

**Table 3**: Counts (log(UFC/mL) of major bacterial groups and Shannon-Weaver diversity index (SDI) in the colostrum and milk samples analyzed in this study.

| | Colostrum (N=17) | | Milk (N=34) | | $\chi^2$ p-value* | p- value† |
|---|---|---|---|---|---|---|
| | n (%) | Median (IQR)§ | n (%) | Median (IQR) | | |
| ***Staphylococcus* spp.** | 6 (35) | 3.28 (2.54 – 4.12) | 8 (23) | 2.74 (2.36 – 3.05) | 0.061 | 0.136 |
| ***Enterococcus* spp.** | 0 | | 10 (29) | 2.81 (2.64 – 3.57) | 0.000 | |
| ***Streptococcus* spp.** | 3 (18) | 2.00 (2.00 – 2.57) | 4 (12) | 3.11 (2.71 – 3.38) | 0.235 | 0.271 |
| ***Lactobacillus* spp.** | 2 (12) | 2.79 (2.40 – 3.19) | 8 (23) | 4.19 (3.77 – 4.47) | 0.041 | 0.068 |
| **Other Gram + bacteria** | 3 (18) | 2.95 (2.93 – 2.98) | 3 (9) | 2.00 (2.00 – 2.80) | 0.063 | 0.506 |
| **Enterobacteria** | 1 (6) | 2.60 | 5 (15) | 2.95 (2.00 – 2.95) | 0.038 | 0.763 |
| **SDI** | 11 (65) | 0.67 (0 – 0.89) | 24 (70) | 0 (0 – 0.78) | 0.450 | 0.505 |

§Results are expressed as median and interquartile range (IQR).

* The Fisher test was used to evaluate differences in frequency of detection between colostrum and milk samples; when required, the Yates correction was applied.

† One-way ANOVA test was used to compare between counts obtained in colostrum and milk samples.

**Table 4**: Concentrations of the immunocompounds in the colostrum (n=15) and mature milk (n=11) samples analyzed in this study

| | Colostrum | | Milk | | $\chi^2$ p-value* | p- value† |
|---|---|---|---|---|---|---|
| | Fr (%)§ | Mean (95%CI) | Fr (%)§ | Mean (95%CI) | | |
| **Immunoglobulins** | | | | | | |
| **$IgG_1$ (mg/L)** | 100 | 87.80 (11.63 – 163.97) | 100 | 10.36 (5.05 – 15.65) | 1.000 | 0.076 |
| **$IgG_2$ (mg/L)** | 47 | 68.04 (-2.92 – 139.00) | 0 | – | 0.045 | 0.229 |
| **$IgG_3$ (mg/L)** | 100 | 2.82 (0.98 – 4.65) | 100 | 0.24 (0.11 – 0.37) | 1.000 | 0.017 |
| **$IgG_4$ (mg/L)** | 100 | 0.98 (0.45 – 1.52) | 100 | 0.29 (0.12 – 0.46) | 1.000 | 0.031 |
| **IgM (g/L)** | 100 | 0.78 (-0.20 – 1.77) | 100 | 21.55 (0.87 – 42.22) | 1.000 | 0.168 |
| **IgA (g/L)** | 100 | 8.98 (0.56 – 17.40) | 100 | 0.68 (-0.63 – 3.99) | 1.000 | 0.133 |
| **Innate immunity** | | | | | | |
| **IL-$1_\beta$ (ng/L)** | 100 | 42.28 (-32.23 – 116.81) | 91 | 0.37 (0.06 – 0.68) | 1.000 | 0.338 |
| **IL-6 (ng/L)** | 100 | 73.97 (18.69 – 129.24) | 91 | 3.27 (1.43 – 5.12) | 1.000 | 0.036 |
| **IL-12(p70) (ng/L)** | 80 | 6.33 (3.78 – 8.89) | 18 | 0.16 (-1.62 – 1.94) | 0.000 | 0.057 |
| **INF-γ (ng/L)** | 53 | 87.95 (18.04 – 157.85) | 0 | – | 0.005 | 0.000 |
| **TNF-α (ng/L)** | 80 | 36.86 (6.39 – 67.32) | 64 | 3.11 (1.09 – 5.13) | 0.012 | 0.083 |
| **Acquired immunity** | | | | | | |
| **IL-2 (ng/L)** | 20 | 2.42 (-1.48 – 6.32) | 0 | – | 0.238 | 1.000 |
| **IL-4 (ng/L)** | 67 | 4.24 (2.43 – 6.05) | 0 | – | 0.000 | 0.000 |
| **IL-10 (ng/L)** | 33 | 1.31 (-0.13 – 2.75) | 27 | 0.89 (-0.54 – 2.31) | 0.354 | 0.582 |
| **IL-13 (ng/L)** ‡ | 60 | 0.80 (0.39 – 0.88) | 82 | 32.32 (6.31 – 37.78) | 0.001 | 0.000 |
| **IL-17 (ng/L)** ‡ | 67 | 126.03 (73.75 – 139.83) | 0 | – | 0.000 | 0.000 |
| **Chemokines** | | | | | | |
| **IL-8 (µg/L)** | 100 | 15.48 (-6.84 – 37.80) | 100 | 0.04 (0.02 – 0.06) | 1.000 | 0.218 |
| **GRO-α (µg/L)** | 100 | 11.50 (5.56 – 17.44) | 91 | 16.41 (2.39 – 30.43) | 0.856 | 0.426 |
| **MCP-1 (µg/L)** | 100 | 7.25 (3.03 – 11.47) | 100 | 0.36 (0.15 – 0.57) | 1.000 | 0.006 |
| **MIP-$1_\beta$ (µg/L)** ‡ | 100 | 0.86 (0.25 – 1.23) | 100 | 0.05 (0.03 – 0.09) | 1.000 | 0.000 |
| **Hematopoietic stimuli** | | | | | | |
| **IL-5 (ng/L)** | 80 | 1.38 (0.59 – 2.17) | 9 | 0.24 | 0.003 | 0.398 |
| **IL-7 (ng/L)** | 100 | 142.64 (-4.38 – 289.67) | 91 | 63.55 (25.57 – 101.52) | 0.856 | 0.365 |
| **G-CSF (ng/L)** | 100 | 107.53 (12.22 – 202.84) | 91 | 21.90 (5.72 – 38.07) | 0.856 | 0.134 |
| **GM-CSF (ng/L)** | 7 | 84.15 | 36 | 51.84 (-12.93 – 116.60) | 0.001 | 0.529 |

Levels of immunocompound were expressed as mean and 95% confidence interval (95% CI) when data were normal distributed and as median and interquartile range (IQR) when not. §Frequency: percentage of samples in which each compound was detected and quantified. *Fisher test was used to evaluate differences in frequency of detection between colostrum and milk; when required, the Yates correction was applied. † One-way ANOVA was used to determine the differences between colostrum and milk when data were normal distributed and Kruskal –Wallis test when they were not. ‡Not normal distributed.