Breast milk polyamines and microbiota interactions. Impact of mode of delivery and geographical location

Running head: Interactions between polyamines and microbiota in human milk

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This study was presented at The Power of Programming 2016 conference in Munich.

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Abstract

Background/Aims: The aim of the present study was to identify and quantify the polyamine levels in human milk from different countries and different mode of delivery, and to investigate their association with breast milk microbes.

Methods: Mature breast milk samples were obtained from 78 healthy mothers after 1 month of lactation from four different geographical locations (Europe, Africa and Asia). Polyamines were determined using HPLC after dansyl derivatization and milk microbiota was obtained by 16S rRNA gene sequencing.

Results: The mean values of polyamines in breast milk were 70.0, 424.2 and 610.0 nmol/dl for putrescine, spermidine and spermine respectively, and 1170.9 nmol/dl of total polyamines. The levels of putrescine were significantly higher in Spain ($p<0.05$) and spermidine levels were significantly higher in Finland ($p<0.05$) compared with other countries. Cesarean delivery had an impact on polyamine levels and it was related to an increase in the putrescine concentration being significant in Spanish samples ($p<0.01$). Furthermore, putrescine levels were correlated positively with Gammaproteobacteria ($r=0.46$, $p<0.001$), especially with *Pseudomonas fragi* ($r=0.40$, $p<0.001$).

Conclusions: The results demonstrate significant effect of geographical variations in human milk polyamine concentrations, with correlation with human milk microbiota composition. These differences may impact on infant development during lactation.

Key Words: polyamines, putrescine, spermidine, spermine, caesarean, breast milk, microbiota, Proteobacteria.

1 **Introduction**

2 Polyamines are organic polycations that are derived from amino acids and are present
3 in all living cells. Representative molecules in mammals include putrescine,
4 spermidine, and spermine. Although polyamines are known mainly for having an
5 essential role in cell growth and proliferation, their functions range from a critical role
6 in mRNA translation, to protection from oxidative damage and activity of ion channels
7 in eukaryotes, to bacterial biofilm formation and specialised roles in the biosynthesis
8 of several natural products [1, 2].

9 The presence of polyamines has been reported in human milk, with higher amounts of
10 spermidine and spermine than putrescine [3-5]. Polyamines are synthesised by
11 lactating mammary tissue [6] and are involved in the hormonal regulation of lactogenic
12 processes in the mammary gland [7], which can explain their presence in milk. A large
13 individual variation in polyamine levels in human milk has been recorded and is related
14 to the age of the mother, ethnic origin, dietary intake of polyamines, and nutritional
15 status, among other factors [3, 8]. The average daily intake of polyamines during
16 lactation has been estimated to be 3.5 $\mu\text{mol/day}$ in breastfeeding children [4].

17 The *in vivo* molecular functions of polyamines are still largely unknown [1], although
18 polyamine ingestion via breast milk seems to have an essential role in gut
19 development and the maturation of associated organs, such as the liver and pancreas
20 [9-11], differentiation and development of the immune system [9, 12, 13], and
21 microbial gut colonisation [14, 15].

22 There are no general recommendations for polyamine intake during lactation, but an
23 increased need for dietary polyamines is suggested during rapid cellular growth stages,
24 such as the neonatal period [16]. In addition, there appears to be a correlation

1 between the low intake of polyamines via breast milk or formula during the first
2 months of life and a higher risk for developing an allergy later in childhood [9, 17].

3 Moreover, human milk is also a source of microbes, which impact gut colonisation
4 during lactation and, therefore, the future health of infants [18]. The microbiota
5 composition in human milk can be influenced by various factors, such as gestational
6 age, lactation stage, maternal health, and mode of delivery [19-21]. Even though the
7 variety of polyamines found in bacteria is higher than in mammals, the two most
8 common and dominant polyamines are putrescine and spermidine [1, 22]. Bacteria
9 control the intracellular content of polyamines by biosynthesis from amino acids and
10 uptake mechanisms, as well as by degradation and efflux processes [23]. Recent
11 studies suggest that polyamines play a crucial role in both mechanisms of bacterial
12 pathogenesis and the beneficial effects of probiotics [23, 24]. Consequently, changes in
13 milk polyamines could be associated with changes in both milk microbiota and infant
14 intestinal microbiota composition and activity. To our knowledge, the impact of milk
15 microbiota on milk polyamine content is not known.

16 The goal of the present study was to identify and quantify the polyamine levels in 1-
17 month-postpartum human milk samples from four different countries. The second goal
18 was to assess whether there was an association between breast milk microbiota and
19 polyamines. The aim was to provide novel information on the interrelationships
20 between maternal and perinatal factors, such as the geographic location and the mode
21 of delivery on the polyamine content of breast milk and their relationship to milk
22 microbiota.

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1 **Materials and Methods**

2 ***Breast milk samples***

3 Breast milk samples were obtained 1 month post-partum from 78 healthy women
4 volunteers practicing exclusive breastfeeding, with a different mode of delivery, and
5 from four geographical locations, including China (10 cesarean deliveries, 10 normal
6 deliveries), South Africa (10 cesarean deliveries, 8 normal deliveries), Finland (10
7 cesarean deliveries, 10 normal deliveries), and Spain (10 cesarean deliveries, 10 normal
8 deliveries), as described by Kumar *et al.* 2016 [20]. The study was approved by the
9 Ethics Committees of the respective participating institutions, Spain (Bioethics
10 Committee of CSIC and the Regional Ethics Committee for Biomedical Research),
11 Finland (Ethics Committee, Hospital District of Southwest Finland), China (Medical
12 Research Board of Peking University), and South Africa (University of Cape Town,
13 Human Research Ethics Committee). All the samples were kept frozen at -20 °C until
14 delivery to the laboratory and then stored at -80 °C until further analysis. To avoid bias,
15 all milk samples were obtained using the same standardised protocol, and they were
16 processed and analysed in a unique laboratory.

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18 ***Analysis of Polyamines by HPLC***

19 Polyamines were analysed at the University of Turku, Finland, with our previously
20 developed method [25], with small modifications based on the method employed by
21 Galitsopoulou *et al.* 2015 [26]. The Shimadzu Prominence HPLC-UV system (Kyoto,
22 Japan) consisted of an SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column
23 oven, a DGU-20A5 degasser, and an SPD-20A UV detector. The analytical column was a

Spherisorb 5.0- μ m ODS2, 4.6 mm $\times$ 150 mm (Ref. PSS831913, Waters Corp., Milford, MA). Detection was performed at 254 nm.

The samples were diluted in sulfosalicylic acid for protein precipitation. After centrifugation at 10,000 $\times$ g for 15 min at 4°C, the supernatants were collected and dansylated by adapting the methods described by Buts *et al.* 1995 [4] and Galitsopoulou *et al.* 2015 [26]. After derivatisation, 200 μ L of L-proline solution (AppliChem, Darmstadt, Germany) was added to remove the excess dansyl chloride [25]. Extraction of dansyl derivatives was performed twice with cyclohexane. 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 0.2- μ m PVDF filters. Samples were run for 30 min according to a gradient method with water and acetonitrile [25]. Each sample was analysed 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 sample preparation, 1,3-diaminopropane (Acros Organics, Geel, Belgium) was used as an internal standard.

Microbial DNA Extraction and Sequencing

Microbial DNA extraction and sequencing by an Illumina MiSeq sequencing platform has been described in a previous study by Kumar *et al.* 2016 [20]. Sequencing data has been submitted to NCBI with SRA accession: SRP082263 and submission ID: SUB1772296.

Data Analysis

Statistical analyses were conducted using SPSS 19.0 software (IBM Corp., Armonk, NY). Differences were considered significant at $p \leq 0.05$. Because of the non-normal distribution, non-parametric tests were used. Comparisons among the data of more than two groups were made by applying the Kruskal–Wallis test, and comparisons between the data of the two groups were made by applying the Mann–Whitney U test. Calypso version 6.1 was used with data normalised by cumulative sum scaling to generate heat maps for Pearson’s correlations between microbial groups and polyamines.

Results

Polyamines in human milk

The median (range) polyamine concentrations in human mature milk samples were 1030.55 (474.4-3518.4) nmol/dl. The predominant polyamine was spermine in almost all samples, regardless of the location or the subjects’ modes of delivery. The median (range) concentrations of polyamines found in breast milk were 21.0 (n.d.-1113.5), 352.5 (142.9-1646.6), and 508.4 (75.6-1593.7) nmol/dl for putrescine, spermidine, and spermine, respectively. Table 1 shows the concentrations of putrescine, spermidine, spermine, and total polyamines in milk samples by country. The polyamine levels varied significantly between the mode of delivery in Spanish samples. The polyamine content and the polyamine profile differed between countries, as shown in Figures 1 and S1 (supplemental data). The level of putrescine was significantly higher in the Spanish samples than in the samples from Finland and South Africa ($p < 0.05$), and the

level of spermidine was significantly higher in the Finnish samples than in the Chinese samples ($p<0.05$). The spermine and total polyamine levels did not differ.

The mode of delivery was associated with some differences in the polyamine profiles (Table 1, Figure S1). The content of putrescine was significantly higher in Spanish cesarean delivery samples than in any other samples ($p<0.01$).

Polyamines and microbiota interactions in breast milk

Pearson's correlation between milk polyamines and milk microbiota are shown as heatmap in Figure S1. Spermidine, spermine, and total polyamines show similar profile without significant correlation with bacterial groups. Only spermidine levels had a positive correlation with bacilli in 97% of the samples ($r=0.24$, $p<0.05$).

Conversely, putrescine levels showed a strong positive correlation with Gammaproteobacteria in 99% of the samples ($r=0.46$, $p<0.001$), and inside this class, with *Pseudomonas fragi* ($r=0.40$, $p<0.001$); and a strong negative correlation with other Proteobacteria (Alphaproteobacteria $r=-0.36$, $p<0.01$; Betaproteobacteria $r=-0.52$, $p<0.001$), Bacilli ($r=-0.54$, $p<0.001$), Clostridia ($r=-0.42$, $p<0.001$) and Actinobacteria ($r=-0.42$, $p<0.001$).

Taking the mode of delivery as factor (Figure S1), the results of caesarian delivery were similar to those reported for all samples together, with a high correlation between Gammaproteobacteria and *P. fragi* and putrescine, whereas the milk samples from mothers who underwent vaginal delivery revealed a different profile. In those samples, the correlation between putrescine and Gammaproteobacteria was highly reduced, although the positive correlation remained with *P. fragi*. In addition, there was a significant negative correlation with Betaproteobacteria for the three studied

polyamines in all of the samples from vaginal delivery: spermine ($r=-0.42$, $p<0.001$), spermidine ($r=-0.39$, $p<0.05$), and putrescine ($r=-0.46$, $p<0.001$).

Discussion

Milk polyamines composition

As has been recently reported by Kumar and coworkers [20], human milk composition can be influenced by environmental factors in both nutritional and bacterial composition. In the present study, we report that these factors also modulate polyamine concentrations in breast milk and their association to milk microbiota. As has been previously reported, the main differences in milk polyamine levels were observed for putrescine and spermidine [3]. This may suggest that putrescine and spermidine in milk may be more susceptible to the influence of environmental factors than spermine. Putrescine levels were also influenced by the mode of delivery.

In spite of the important role of breast milk polyamines on early development, there are no recommendations for daily intake of polyamines for infants. Several studies have been published evaluating the polyamine concentrations in human milk, but reported results were highly different because of the different methodologies; milk collection, storage, and analysis; and high interindividual variability. All the previous studies support the conclusion that putrescine is less abundant in human milk comparing with spermidine and spermine, and our study agrees with earlier reports by Romain *et al.*, 1992 [5], Dorhout *et al.*, 1996 [27], and Atiya Ali *et al.*, 2013 [3]. Regarding spermidine and spermine in human milk, several reports show more heterogenic data. Our results were similar for spermidine to those reported by Romain *et al.*, 1992 [5], Dorhout *et al.*, 1996 [27], and Atiya Ali *et al.*, 2013 [3]; and higher than

1 those reported by Buts *et al.*, 1995 [4] and Pollack *et al.*, 1992 [28]. For spermine, our
2 results are similar to those reported by Romain *et al.*, 1992 [5] and higher than those
3 reported by Buts *et al.*, 1995 [4], Pollack *et al.*, 1992 [28], Dorhout *et al.*, 1996 [27], and
4 Atiya Ali *et al.*, 2013 [3]. Therefore, common methodologies are required to assess and
5 compare the concentrations of polyamines in human milk.

6 This study demonstrates that, even when the great interindividual variability is
7 considered, the geographical location is more important a factor for polyamine
8 composition of human milk than the mode of delivery. Polyamine concentrations in
9 milk are directly correlated with dietary intake of polyamines [3]. Consequently, the
10 differences found between countries might be related to different dietary patterns.

11 It has been reported that the probability of developing a food allergy during the first
12 month of life can increase by 80% if the content of spermine in milk or formula is lower
13 than 0.4 mg/l, and the probability is practically nonexistent when the content of
14 spermine is higher than 2.6 mg/l [9]. Although spermine is the most active polyamine
15 for several biological processes, polyamines can be metabolically interconverted, and
16 the total content of polyamines should be taken into consideration in future studies.

17 In this study, the milk samples from five mothers from different countries had a
18 content of spermine lower than 0.4 mg/l (four from caesarean delivery), and in six
19 mothers this was higher than 2.6 mg/l (four from normal delivery). This suggests that
20 approximately 6.5 % of the mothers in this study, mainly who had delivered via
21 caesarean, had such a polyamine composition, which may significantly increase the risk
22 of developing allergic disease in infants.

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Milk polyamines and microbiota

The combination of a polyamine profile and breast milk microbiota shows the association of polyamines with specific bacterial groups.

Polyamines participate in several physiological processes in bacteria, including cell growth, biofilm formation, and free-radical scavenging [22]. In bacteria, the internal concentration of polyamines is regulated metabolically and through membrane transport [1, 23, 29]. Several bacteria isolated from human milk have been identified as putrescine producers, which may be a species-level characteristic [30, 31]. In addition, alterations in microbiota of the mammary gland may be related with changes in the polyamines profile, such as in the case of mastitis-affected samples, which show higher levels of putrescine in milk compared to healthy breast milk samples [32].

Our study suggests that putrescine is positively correlated with Gammaproteobacteria, mainly with *Pseudomonas fragi*. Recently it has been discovered that caesarean section mothers have higher levels of Proteobacteria class in milk [20] and higher levels of *P. fragi* (unpublished data) than mothers who delivered normally. Polyamine production was reported in the Proteobacteria group with the generation of putrescine, spermidine, and spermine, and also included fewer common polyamines, such as homospermidine, with the production of one or several of these polyamines being genera-dependent [31]. The origin of milk polyamines is not clear, but taken together, the fact that putrescine is an important bacterial metabolite, and the strong correlation with Gammaproteobacteria, putrescine in milk may have a bacterial origin, while spermidine and spermine may be consequences of the high metabolic activity in the mammary gland.

1 The interplay among polyamines and the development of initial microbiota is not
2 known. A study with lactating mice showed that the supplementation of a commercial
3 infant formula with different amounts of polyamines induces changes in microbial
4 colonisation patterns along the gastrointestinal tract, from the mouth to the large
5 intestine [14]. Similar processes may take place in humans. Therefore, different breast
6 milk polyamine profiles may be related to different milk microbiota, impacting on the
7 development of infant gastrointestinal microbiota with consequences to infant health.

8 9 **Conclusions**

10 Several recent studies on the function of polyamines during lactation have brought
11 new insight regarding their importance in infant development and health. To our
12 knowledge, this is the first study reporting the correlation between the main
13 polyamines and specific microbial groups in human milk.

14
15 The present study demonstrates that there are differences in the composition of
16 polyamines in breast milk in different geographic regions, which may be associated
17 with differences in infant development. The impact of mode of delivery on milk
18 polyamines seems to also be different within a geographical location.

19
20 Further studies are required to determine whether the observed differences in milk
21 polyamines may be used as indicators of milk bacterial composition and its impact on
22 infant development.

1 **Statement of Authorship**

2 The work was initially designed by HK, ET, MC, KL, BY, EI, and SS. Data contributors
3 were CGG, IGM, JPS, HK, ET, YZ, and MC. The manuscript was drafted by CGG, MC and
4 SS, edited and approved by all authors.

5

6 **Disclosure Statement**

7 All authors have no conflict of interest.

8

9 **Acknowledgements**

10 C. Gómez-Gallego is a recipient of the Seneca Postdoctoral Grant from the Seneca
11 Foundation, the Regional Agency of Science and Technology of the Region of Murcia
12 (funded by the Education and Universities Council - Autonomous Community of the
13 Region of Murcia, Spain). M.C. Collado is a recipient of an European Research Council
14 (ERC) Starting Grant 639226-MAMI. The funders had no role in the design, analysis, or
15 writing of this article. Sequencing carried out at CPGR, South Africa, thanks to funding
16 from the National Institutes of Allergy and Infectious Disease 1U01AI110466-01A1,
17 H3Africa Initiative. Chinese group acknowledges the support from Key Projects of
18 Beijing Science & Technology (D141100004814002), and Natural scientific foundation
19 of Beijing (Z140001).

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1 **Legends**

2 **Table 1.** Concentration of polyamines in milk

3

4 **Figure 1.** Box and whisker plot comparing levels of polyamines in breast milk between
5 different countries. Different letters indicate statistically significant differences at the
6 level of $p < 0.05$.

7

8 **Figure S1.** Comparison of percentage relative abundances of polyamines taking
9 country (A1) and mode of delivery (A2) as factors. Heatmap to show Pearson's
10 correlation between polyamine and microbiota composition at class level (B1) and
11 species level (B2). Heatmap to show Pearson's correlation between polyamine and
12 microbiota composition in normal delivery samples (C1, C2) and caesarian samples (C3,
13 C4) at class level (C1, C3) and species level (C2, C4). PUT – putrescine; SPD –
14 spermidine; SPM – spermine; Total – total polyamines.