Research article

Analysis of gene expression from human breastmilk cells: A comparison between low and high producers, and the influence of anxiety and depression on milk production, gene expression and bacterial production

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ABSTRACT

Background: Breastmilk is considered the gold standard of infant nutrition. Many mothers have difficulty with breastfeeding and over 50% of women stop due to perceived low production.

Aims and methods: Our study compared gene expression in 8 samples of low and high producers of milk. All subjects were administered GAD-7 and PHQ-9 questionnaires. Low-producers were all found to have more depression and anxiety compared to high-producers.

Results: We did not find significant differences between gene expression between low and high milk producers. Only 5 of 8 samples contained a significant number of human cells. We did find differences in the amount of various bacterial populations.

Conclusion: Our results indicate that gene expression in breastmilk is complicated by collection methods. We recommend that even though some women produced less than 600 ml of milk over a 24-hour period of time, due to the nature of the bacteria found in milk they try to breastfeed as much as they can for the health benefits of their infants. the rich bacterial diversity in all patients including the low producers strongly suggests that even women producing lesser quantities of milk confer their children numerous benefits by breastfeeding them.

1. Introduction

Breastmilk is a complex biological fluid that can provide optimal nutrition for infants while imparting significant health benefits to mothers [1, 2]. The American Academy of Pediatrics (AAP) and World Health Organization (WHO) recommend exclusive breastfeeding for the first 6 months of life and ideally until age 1 or beyond as long as the mother and infant are willing [3, 4]. Lactation is a dynamic process, not completely understood, involving maternal genetics, diet, and environmental exposures in addition to infant demand [5]. Successful lactation and exclusive breastfeeding are adversely affected by prior breast surgery [6], and breast hypoplasia [7]. Recently we have gained a more comprehensive understanding of psychological factors such as anxiety and depression, that may adversely affect milk production and the ability to breastfeed [5]. Further, low milk-production in itself can cause mood disorders in a nursing mothers [8].

It is estimated that 10–15% of women report not producing enough milk [9, 10]. This can lead to failure to thrive [11, 12], hypernatremia [13], and nutritional deficiencies [12]. In the U.S., almost 40–50% of women stop nursing due to perceived lack of supply or perception of the baby not being satisfied with breast milk alone [10].

In early pregnancy, lactogenesis is largely controlled by reproductive hormones which drive mammary gland development, and mechanisms which regulate nutrient transport, milk production, and secretion from the mammary glands. During mid-pregnancy, lactogenic genes drive the differentiation of the mammary gland into secretory mammary epithelial cells (MECs) which coordinate factors influencing the constituents of milk before it can be secreted post parturition. Therefore, various recent studies are examining RNA obtained from human milk to characterize MEC-specific gene expression and identify genes strongly correlated with lactogenesis [14].
Milk secretion from the MECs is under hormonal control and is normally proportional to milk removal from the mammary glands [15]. Initiation of lactation is normally stimulated through infant suckling but once it is established, maternal genetics, diet, and environmental factors play a large role in the composition and continuity of milk production and the response of the breast to the infant's milk demand [16].

Maternal genetic variants in hormone receptor signaling and nutrient transport-related genes not only explain differences in nutrient contents in breastmilk [16, 17] but recent studies examining the milk cell transcriptome throughout the lactation cycle attempt to also provide insight into understanding differences between over-producing and under-producing lactating women [14].

Depression and anxiety also affect postpartum hormones and are associated with early cessation of breastfeeding [15]. Previous studies have shown disrupted lactation in mothers with depression [18] differences in oxytocin response between patients with and without depression [19] and other negative infant-feeding outcomes, resulting in decreased maternal initiative to breastfeed [20]. Additionally, perinatal depression and failed lactation share a common pathophysiological basis in terms of their neuroendocrine mechanism [8]. Since the gene expression in breastmilk greatly varies among women based on their demographics, gestational age at delivery, as well as maternal BMI [21], our study wanted to examine whether anxiety and depression could alter gene expression and bacterial composition of human milk, ultimately having nutritional and immune consequences to infants.

Therefore, our study wanted to evaluate the correlation between milk production and depression and anxiety as measured with the PHQ-9 and GAD-7 scales (previously validated) to see if there was a correlation between milk production, anxiety and depression and gene expression. We also wanted to look at the differences in bacterial production between low and high milk producers.

2. Methods

2.1. Subjects

Four women were low-producers which they reported as producing less than 600 ml of breast milk in 24-hours. Four other women produced over 1500 ml per 24-hour period and were considered to be high-producers. Mothers were all at peak lactation, all having met the criteria for term delivery of infants, delivery of singletons, and age of infant between 30-60 days at the time of milk collection. This study was approved by the University of California, Los Angeles Ethics and Institutional Review Board and a written informed consent was obtained from all participants. Each subject received a $20 gift card for participating in the study.

2.2. Milk samples

Milk was collected at home, after a single pumping session after sterilizing pumping equipment and collected in a sterile container. Mothers were instructed to collect milk at a time of no clinical signs of engorgement, or any infection. 30 ml of milk was collected by each subject. Mothers were instructed to wash their hands with soap and water for 30 s prior to milk collection. The samples were shipped to the lab in liquid nitrogen and stored in a -80 °C until processing.

2.3. RNA preparation

Total RNA was isolated using Qiagen RNeasy Micro kit and following the instruction. The integrity of the isolated RNA was examined by the Agilent 4200 TapeStation System. Libraries for RNA-Seq were constructed with KAPA RNA HyperPerc Kit to generate strand-specific RNA-seq libraries. The workflow consists of ribosome RNA (rRNA) depletion, RNA fragmentation and double-stranded cDNA generation using a mixture of random priming, followed by end repair to generate blunt ends, adaptor ligation, strand selection, and PCR amplification to produce the final libraries. Amplified libraries were quantified by Qubit dsDNA HS (High Sensitivity) Assay Kit, and quality-checked by the Agilent 4200 TapeStation System. Different index adaptors were used for multiplexing samples in one sequencing lane. Sequencing was performed with Illumina HiSeq 3000 sequencer to produce 50 base-pair single-end reads (1 × 50 bp).

2.4. Differential gene expression analyses

RNA-seq libraries were sequenced 1 × 50bp on an Illumina HiSeq3000 system, yielding 30–78M reads per sample for 8 samples. Reads were quality checked with FastQC v0.11.8 [22] filtered with Trimmomatic v0.38 [23], and aligned to the GRCh38 human reference genome with STAR v2.6.1d [24] using GENCODE H31 annotations, retaining uniquely mapped reads with less than 5 mismatches. Three samples had less than 1M reads aligned to the human genome and were not used in subsequent analyses. Gene expression was quantified for 2 under-producer and 3 over-producer samples using HTSeq [25] over exons in union mode and analyzed using the R package EdgeR [26, 27]. Gene expressions were TMM-normalized and differences between groups were assessed using the GLM approach. Sequence data files have been deposited at the National Center for Biotechnology Information Sequence Read Archive under the BioProject accession number PRJNA768101.

2.5. Bacterial diversity analyses

Using Kraken2 [28] and the “minikraken2.v2.8GB.201904” taxonomic database, taxonomic assignments could be obtained for 23–32% (across 8 samples) of the filtered RNA-seq reads. Community composition estimates at the genus level were then derived with Bracken [29].

3. Results

Clinical and demographic characteristics of the study participants (n = 8) are described in Table 1. Mothers were on average 32 years of age, most of them were Caucasians and all delivered vaginally. The average age of the infants at the time of milk collection was 44 days. The average BMI of the mothers was 25. Mothers were not taking any medications besides over-the-counter vitamins. All participants were non-smokers and denied any alcohol or drug use.

The participants depression and anxiety as measured with the PHQ-9 and GAD-7 scales are described in Table 2. All high-producers scored 0 on the GAD-7 Scale and the PHQ-9 scale. They did not meet the criteria for a screening diagnosis of anxiety or depression. The low-milk producers all scored between 6-9 on the PHQ-9 questionnaire (Table 1). This is indicative of mild depression. The low-producers all scored between 7-9 on the GAD-7 questionnaire, which indicates mild anxiety (Table 1). As shown in Figure 1, the low-producers in our patient cohort, more likely to have anxiety and depression and showed the absence of Streptococcus and Staphylococcus, while the high-producers were positive for both these species. Additionally, three of out the eight patients were positive for pseudomonas, and two of those patients were low-producers.

Evaluation of mRNA expression was completed on all 8 samples collected from 8 subjects. Only 5 of 8 samples contained human cells (Figure 2). Further, deconvolution analyses were performed on the 5 samples that had detectable number of human cells. The predictions were similar among samples consisting of various cell types (Figure 2). Cell type composition of the six samples were predicted using the Gene Expression Deconvolution Interactive Tool (GEDIT [30]). GEDIT utilizes gene expression data from cell type reference profiles and from unknown mixtures to infer cell type content via linear regression. The reference data used here was the BlueCode matrix (available here: https://github.com/BNadel/GEDIT/tree/master/ReferenceMatrices), which represents a combination of data from the ENCODE and BLUEPRINT projects [31, 32]. Default settings were used for GEDIT. This experiment was approved by the UCLA IRB committee.
4. Discussion

The association between milk production and anxiety and depression explored in previous studies was further demonstrated in our patient cohort, which showed a strong correlation between under producers and anxiety and depression in terms of their PH9-Q and GAD-7 scores. Another goal of our study was to examine the gene expression and bacterial production between the patients. Notable differences in gene expression were not found between the low-producers and high-producers in our patient population. This is likely because of the small sample size (only 3 low-producers and 2 samples from high-producers, were used in differential expression analyses). The protective qualities of breast milk are not limited to immunoglobulins but also result from its natural flora including Staphylococci, Streptococci, Micrococci, Lactobacilli, Pseudomonas, and Enterococci [33]. The bacterial diversity observed was substantial. However, it should be noted that due to the small sample size the differences are qualitative and not statistically significant. We cannot also exclude collection method differences and error despite each mother being instructed individually on milk collection methods. Finally, that the patterns we see may be caused by inter-patient variability in the abundancies of human (epithelial or immune) cell populations, bacterial cell populations (even in absence of mastitis), or both.

Table 1. Demographic information for patient cohort.

| Genetic Study Number | Milk Production | Age | Ethnicity | BMI | Pregnancies | Age of Child (days) | Live Children | Number Delivered | Delivery |
|----------------------|-----------------|-----|-----------|-----|-------------|---------------------|---------------|------------------|----------|
| S1                   | high-producer   | 28  | Caucasian | 30  | 1           | 34                  | 1             | 1                | Vaginal  |
| S2                   | high-producer   | 32  | Caucasian | 26  | 2           | 45                  | 2             | 1                | Vaginal  |
| S3                   | high-producer   | 38  | Caucasian | 26  | 4           | 51                  | 3             | 1                | Vaginal  |
| S4                   | high-producer   | 32  | Caucasian | 23  | 2           | 39                  | 2             | 1                | Vaginal  |
| S5                   | Low-producer    | 30  | Caucasian | 21  | 2           | 33                  | 2             | 1                | Vaginal  |
| S6                   | Low-producer    | 37  | Asian     | 20  | 3           | 47                  | 3             | 1                | Vaginal  |
| S7                   | Low-producer    | 28  | Caucasian | 24  | 2           | 44                  | 2             | 1                | Vaginal  |
| S8                   | Low-producer    | 33  | Caucasian | 28  | 3           | 55                  | 1             | 1                | Vaginal  |

Low-producer = <600 ml of breastmilk produced in 24-hrs. High producer = >1500ml of breastmilk produced in 24-hrs.

Table 2. PHQ-9 management summary and GAD-7 scale.

| Score | Depression Severity PHQ-9 | Anxiety Severity GAD-7 |
|-------|---------------------------|------------------------|
| 0–4   | Minimal or None           | -                      |
| 5–9   | Mild                      | Mild anxiety           |
| 10–14 | Moderate                  | Moderate anxiety       |
| 15–19 | Moderately Severe         | Severe anxiety         |

Figure 1. Heat map of bacterial population across patient samples indicated as a fraction of the total reads per sample.
The deconvolution of the human cells detected in the 5 milk samples consisted of epithelial, immune cells including B-cells, T-cells, NK cells, macrophages and neutrophils, which is consistent with previous findings. We also detected eosinophils, platelets and reticulocytes. The myriad of cells of immune cells in breastmilk that were collected by asymptomatic mothers, does support the unique immune properties of human milk.

5. Conclusion

The probiotic bacteria in human milk colonizes the infant gut and helps establish the infant microbiome and prevent colonization by more severe pathogens [34]. Hence, the bacterial diversity in all patients including the low-producers strongly suggests that even women producing lesser quantities of milk confer their children numerous benefits by breastfeeding them and hence should be encouraged to continue doing so even if it is a small amount.

Declarations

Author contribution statement

Stephanie Canale, Matteo Pelligrini: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Renuka Ramanathan: Analyzed and interpreted the data; Wrote the paper.

Nicolas C. Rochette, Brian B. Nadel: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Melissa Gee: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at National Center for Biotechnology Information Sequence Read Archive under the Bioproject accession number PRJNA768101.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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