Developing a Bacterial DNA Extraction and PCR Protocol to Study the Breastmilk Microbiome

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Background:

The human milk (HM) microbiome is an essential factor in shaping breastfed infants' immune systems. Previous research studies use frozen breastmilk samples and remove the fat and skim layers from the sample following centrifugation and extract DNA from the remaining cell pellet. This DNA is then used in assessing microbiome composition using 16S rRNA gene sequencing. In doing so, these studies neglect to identify bacterial species that did not survive a freeze-thaw cycle intact, or species associated with the fat fractions. This could skew the resulting microbiome composition analysis and the estimate of infant bacterial exposure from HM. In addition, most researchers use commercial DNA extraction and purification kits which are not optimized for low biomass samples and may not be the most effective extraction method and additionally lead to bacterial DNA contamination.

Objective:

The aim of this study is to develop a protocol to study the complete HM microbiome, by extracting bacterial DNA from whole milk samples for sequencing.

Design/Methods:

Extraction: Based on problems identified in previous work with commercial extraction kits, a modified protocol of phenol-chloroform extraction along with bead beating was used to extract DNA from whole milk. Different volumes of milk sample were tested, as well as whole vs. pelleted milk.

DNA Concentration and Purification: The use of a PCR product DNA purification column vs. no column was tested with extractions. Two brands of commercially available purification columns (Qiagen and Zymo) were tested for innate inhibitors, each using three different eluents: water, the provided elution buffer (EB), and TrisEDTA (TE). The columns were tested for innate PCR inhibitors by spiking increasing volumes of elution with 30 pg of Staphylococcus subsp. Aureus UAMS-1 DNA, and performing PCR for the full-length bacterial 16S gene. PCR products were visualized on a 1.5% agarose gel.

*PCR Amplificatio*n: A literature review of possible PCR inhibitors inherent to the extraction was conducted. Calcium and digestive enzymes present in the milk could potentially inhibit PCR, if not eliminated in the extraction. To counteract these potential effects, increased concentrations of magnesium chloride ($MgCl_2$) from 1.5 to 5mM and the addition of Bovine Serum Albumin (BSA) (.3%) to the PCR reaction, in varying combinations, were tested.

Results:

Extraction: Different volumes of whole milk still yielded a similar amount of DNA yield per mL of milk. Phenol chloroform extraction with whole milk resulted in a DNA yield of ~500pg DNA/mL milk while pelleted milk yielded ~30pg DNA/mL milk. Use of a column resulted in a lower DNA yield (~50pg/mL vs. ~500pg/mL), but less PCR inhibition.

DNA Concentration and Purification: Elution of commercial DNA purification/concentration columns with TE resulted in complete inhibition of PCR. Elution with either water or EB resulted in limited inhibition. Both commercial columns showed inconsistent degrees of bacterial DNA contamination.

PCR Amplification: Adding a higher concentration of $MgCl_2$ to the PCR partially ameliorated the PCR inhibition innate to the milk extraction. BSA did not counteract PCR inhibition.

Conclusion/Future Direction:

Phenol Chloroform extraction of whole milk provides better DNA yields than from pelleted milk. Magnesium Chloride overcomes some the PCR inhibitors innate to the milk extraction. Despite inhibition from purification columns, PCR products of milk extractions run through these columns and eluted in EB, visualize much better on a 1.5% agarose gel, indicating that the use of a column is still important for purification of the extraction. The next steps will be to test if these protocol alterations result in a DNA yield that will support sequencing. From there, 16S sequence based microbiome composition of pelleted milk will be compared to that of whole milk to determine if whole milk samples are a more representative sample of the milk microbiome.