Assessment of Viable Bacterial Communities in Fresh and Stored Human Milk

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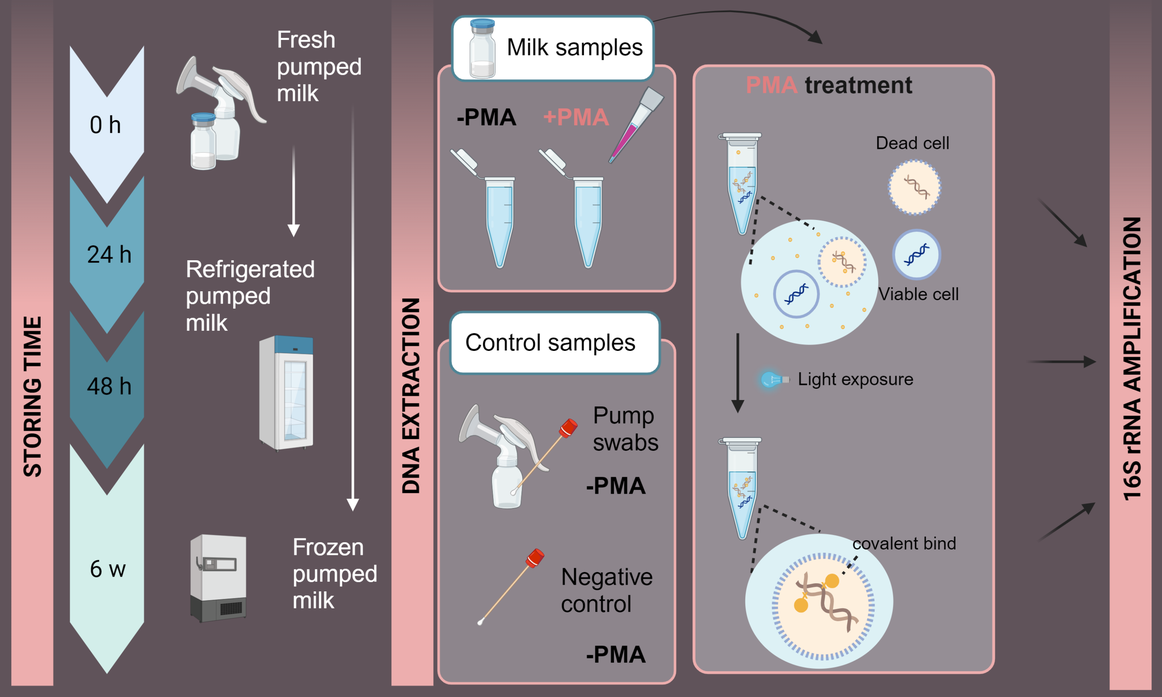
BACKGROUND

Understanding the viable bacterial community in human milk is crucial for assessing its health effects on both mother and infant. While expressing and storing human milk are common practices, there is limited information on how these practices influence the abundance, diversity, and viability of the milk's bacterial community. To address this gap, **our main goal was to characterize the profile of viable pumped human milk bacteria and investigate their survival under various storage conditions.** To achieve this, we focused on differentiating DNA from living and dead cells using propidium monoazide (PMA) in the human milk bacterial populations. This dye binds to DNA, preventing PCR amplification from non-viable cells, thus ensuring that only viable bacterial DNA is detected, providing a clearer insight into the true composition of the milk's bacterial ecosystem.



METHODS

We conducted a metagenomic observational study involving lactating mothers (n=12) who provided freshly pumped milk samples. These samples were stored under various conditions (fresh, refrigerated at 4°C, frozen at -20°C) and threated with PMA to identify non-viable cells. The extracted DNA from each sample was subsequently analyzed using 16S rRNA amplicon sequencing on the Illumina platform.

RESULTS

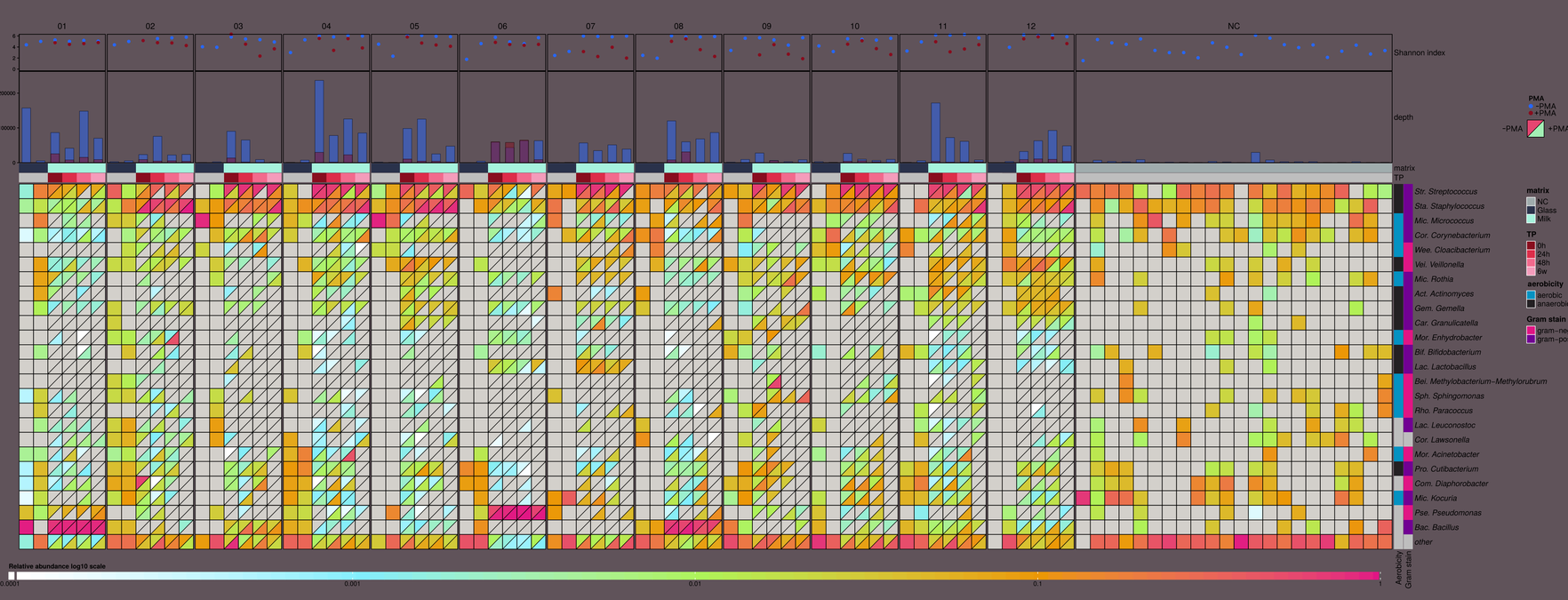


Figure 1. Heatmap of bacterial composition (genus level) of fresh human milk samples, human milk samples stored at different conditions and durations, and swab control samples taken from pump equipment and negative controls.

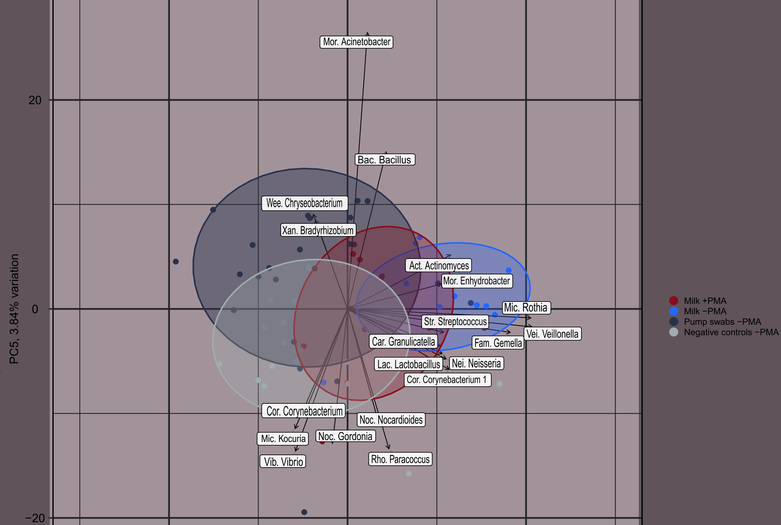


Figure 2. Principal Component Analysis (PCA) at the genus level on human milk samples without any additional treatment (-PMA, n = 48) and human milk samples treated with propidium monoazide (+PMA, n = 48) in comparison to -PMA control samples (negative controls – DNA-free water, n = 24, and swabs from pump equipment, n = 24).

1) The study revealed that human milk samples treated with PMA showed a decrease in detected bacterial reads, suggesting a significant presence of non-viable bacteria, consistent with previous research indicating variability in the microbiota profiles of human milk but general similarity to established microbiome studies.

2) The alpha diversity and presence of *Streptococcus* decreased significantly in PMA-treated samples in fresh human milk samples.

3) Contamination from reagents and breast pump equipment was identified, impacting the microbiome analysis of human milk, with common contaminants being *Diaphorobacter* and *Kocuria*.

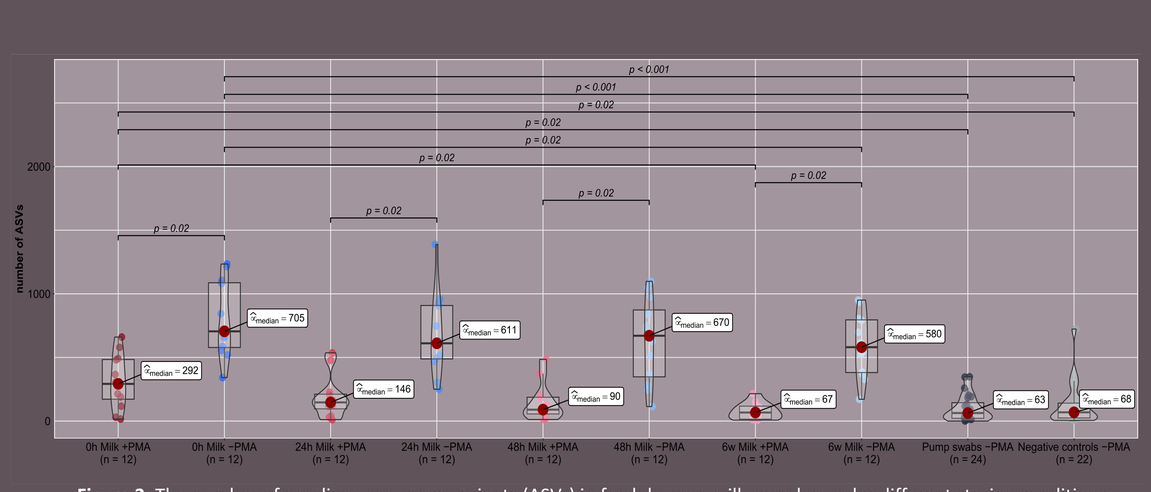


Figure 3. The number of amplicon sequence variants (ASVs) in fresh human milk samples under different storing conditions. Human milk samples were kept at 4°C for 24 and 48 hours, and at -20°C for 6 weeks. Furthermore, samples were treated with propidium monoazide (+PMA) to distinguish the presence of viable bacterial genera. In contrast, samples without PMA treatment (-PMA) exhibit both viable and non-viable bacterial genera.

4) Storage conditions such as refrigeration or freezing did not significantly alter the diversity of bacteria in human milk, though PMA treatment indicated a reduction in viable bacterial diversity over time.

5) *Staphylococcus* genus significantly increased ($p < 0.05$) in +PMA samples over 6 weeks at -20°C. Genus *Streptococcus* significantly decreased ($p < 0.05$) over 6 weeks at -20°C compared to fresh +PMA milk samples.

SUMMARY

The use of PMA has enabled characterization of the viable bacterial profile of fresh human milk where a significant proportion of the bacterial DNA detected in fresh milk originated from non-viable cells. Our findings reveal that while cold storage does not significantly alter the overall diversity of bacteria within human milk, freezing does impact the viability and abundance of certain bacterial genera, such as *Streptococcus* and *Staphylococcus*. The presence of contaminants from pumping equipment and reagents highlighted the need for standard collection and processing protocols. This study emphasizes the need to consider how pumping and storing practices affect the bacterial quality of human milk.

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