Introduction

Human milk is composed of multiple immune factors which interact to form the “immune system of milk” [1]. These components include monocytes, macrophages, antibodies, communication molecules (cytokines), antimicrobial proteins, and commensal microbes.

The immune system of milk is critical to understanding connections between maternal and infant health. The immune system of milk may both protect infants against infectious disease and guide their immune development [2, 3].

First, we identified the optimal culture vessel and duration of incubation for stimulating measurable immune responses in whole human breast milk in vitro. Our priorities were to develop a protocol that:

1) Which cytokine(s) are most readily detectable in stimulated milk specimens?
2) Which is the optimal incubation vessel for stimulating measurable immune responses: borosilicate glass tubes or 6-well, plastic suspension plates?
3) What is the optimal incubation duration for stimulating measurable immune responses?
4) Which gastrointestinal bacteria prompt the greatest response?

Methods

Preparation of milk specimens

Milk specimens were prepared for use in stimulating immune responses in vitro. We employed a variety of measures to reduce contamination of milk with microbes and designed these to eliminate potential false-positives. Milk specimens were cultured in either vessel was suitable. We prefer 6-well plates for practical concerns, such as avoiding the dangers of broken glass, minimizing milk spillage, and stackability. Were minimizing cost the priority, our findings support use of glass culture tubes.

Duration of incubation: Specimens were placed in a glass desiccator. Tea light candles were used to reduce the presence of oxygen within the sealed desiccator, and the desiccator was place in an 37° C incubator.

We tested incubation durations of 24, 48, and 72 h.

Cytokine concentrations (IFN-γ, IL-4, IL-6, IL-10) in baseline and incubated specimens were estimated with the QuanSys 4-plex high sensitivity cytokine enzyme immunoassay.

Increases (from Baseline) were evaluated assayed to evaluate differences across incubation vessels and durations.

Findings

Interleukin-6 responses to stimuli were characterized as ratios of stimulated IL-6 responses to baseline. These are usually smaller than responses to bacterial stimuli.

Gastrointestinal bacterial stimuli: We expanded the protocol to include four species of gastrointestinal bacteria which the immune system of milk is very likely to encounter:

- *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028)
- *Escherichia coli* (ATCC 51813)
- *Bifidobacterium breve* (ATCC 15700)
- *Lactobacillus acidophilus* (ATCC 314)

Among specimens stimulated in vitro with one of these gut bacteria:

- IL-6 could be quantified in 59.13% of specimens
- IL-10 could be quantified in 87.00% of specimens
- IFN-γ could be quantified in 20.00% of specimens

If undetectable specimens are considered to have the value of the lower limit of detection of the assay, the distribution of Baseline, Negative, *Salmonella, E. coli, Bifidobacterium, and Lactobacillus IL-6 concentrations is shown in Figure 5.

**Interleukin-6 responses to stimuli were characterized as ratios of stimulated IL-6 responses to baseline.**

Conclusion:

- Milk in vitro immune responses can occur in the absence of an added stimulus. These are usually smaller than responses to bacterial stimuli.
- Interleukin-6 responses were observed to all gut bacteria evaluated. Responses to *Salmonella* were generally larger.
- Interleukin-10 and interferon-γ responses occurred less frequently than did IL-6 responses.
- This method provides a field-friendly, affordable way to characterize pro-inflammatory activity of the immune system of milk.

Acknowledgments: We are indebted to study participants and their families for their time and patience. Funding was provided by Binghamton University and the Wenner-Gren Foundation.

References:

[3] Binghamton University, SUNY