Protocol development and quantification of immune response in bacterially stimulated human milk

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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].

We developed a protocol to measure milk immune activity *in vitro* to microbial agents, including pathogens and commensal microbes. Responses to these microbes were characterized with cytokines:

- Interferon-γ (IFN-γ; a promoter of type I immune responses)
- Interleukin-4 (IL-4; a promoter of type II immune responses)
- Interleukin-6 (IL-6; a pro-inflammatory cytokine)
- Interleukin-10 (IL-10; an anti-inflammatory cytokine)

Here, we describe the development of the protocol for describing immune responses in whole human breast milk *in vitro*. Our priorities were to develop a protocol that:

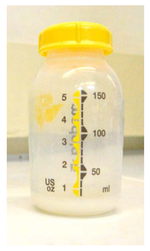
- generates results interpretable at the level of the immune system of milk
- is practical for population-based, international research

Some of the questions we addressed in protocol development were:

1) Which cytokine(s) are most readily detectable in stimulated milk specimens?
2) Which is the optimal incubation vessel to 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

First, we identified the optimal culture vessel and duration of incubation with two stimuli known to induce cytokine production in whole milk upon *in vitro* stimulation, lipopolysaccharide (LPS) and *Mycobacterium bovis* Bacille Calmette-Guérin. We then expanded to include 4 bacteria likely to be found in the infant gut.



Breastfeeding women in the Binghamton, New York, area provided ~1 oz milk, expressed with an electric breast pump.

Within 4 h of expression, ~1.5 ml of each specimen was separated by centrifugation, and the aqueous portion was extracted and frozen, for the baseline specimen.

2 ml of specimen was combined with 1 ml mammalian cell culture medium [RPMI 1640 (Lonza BioWhittaker) with L-glutamine (Gibco, 110 mg/l), pyruvate (Lonza BioWhittaker, 292 mg/l), and penicillin-streptomycin (Gibco, 100 U/ml)] in each of the following conditions:

- Cell culture medium alone: the unstimulated "negative control"
- Lipopolysaccharide (LPS) isolated from *E. coli* (List Biological; 200 µg/ml)
- *M. bovis* Bacille Calmette-Guérin (BCG; TICE BCG, Merck; 2% of vaccine concentration, or 2-16 x 106 CFU/ml)
- *Salmonella enterica* (Microbiologics 0363L; ATCC 14028)
- *Escherichia coli* (Microbiologica 0791E3; ATCC 51813)
- *Bifidobacterium breve* (Thermo Scientific Culti-Loops R4606801; ATCC 15700)
- *Lactobacillus acidophilus* (Thermo Scientific Culti-Loops R4603050; ATCC 314)

Incubated specimens were then separated by centrifugation and the aqueous portion extracted and frozen until assay.

Culture vessel: Specimens were incubated in glass culture tubes (which are more economical) and sterile, individually packaged 6-well suspension plates (which are safer, but more costly), to compare cytokine production across vessels.

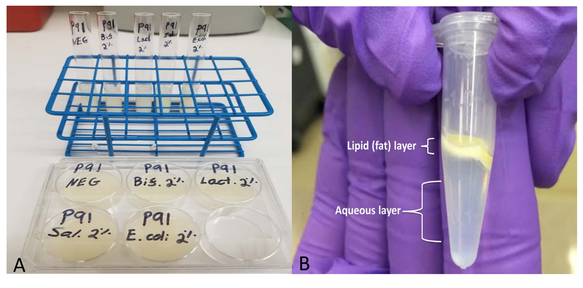


Figure 1. (A) Prepared milk/ bacteria dilutions in either glass culture tubes or suspension plates. (B) Centrifuged milk sample before the aqueous layer is removed and frozen.

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 duration.

Findings

- Interleukin-6 was most frequently quantifiable.
- Interleukin-4 was rarely quantifiable; it was not considered in this analysis.

Negative controls (Figure 2):

- Immune responses (increases in cytokines) to the Negative (unstimulated control) condition were apparent in many of specimens
- We employed a variety of measures to reduce contamination of milk with bacteria or endotoxin (equipment was cleaned and sterilized after each use, participants were asked to use sanitizing wipes on their hands and breasts before pumping).
- Immune responses to the "Negative control" may result from milk's natural composition which are inherently active.

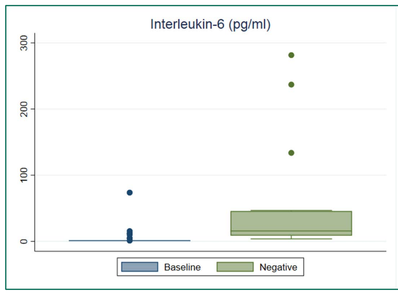


Figure 2. Estimated interleukin-6 concentrations in Baseline and Negative control (incubated without addition of a bacterial stimulus) specimens. Negative control specimens frequently exhibited IL-6 responses (increases in IL-6 compared to Baseline). We limited opportunities for contamination of milk and so conclude that these responses reflect real characteristics of the immune system of milk (e.g., "background" immune activity or responses to commensal microbes).

Incubation vessel:

- Cytokine responses in glass culture tubes and plastic 6-well plates were similar (for LPS, Spearman's ρ: 0.9081 for IL-6 and 0.7559 for IFN-γ; for BCG, Spearman's ρ: 0.6988 for IL-6 and 0.9592 for IFN-γ; **Figure 3**).
- We concluded incubation 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.

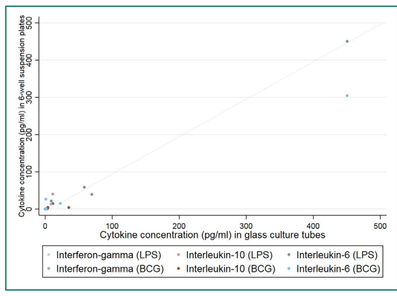


Figure 3. Cytokine concentrations estimated in specimens incubated with *M. bovis* BCG (BCG) or lipopolysaccharide (LPS) in **glass culture tubes** (x) or 6-well **plastic suspension plates** (y). The reference line has a slope of one. Cytokine production was generally higher in plates, but not substantially so. Use of either plates or tubes is supported.

Incubation duration (24, 48, 72 hours):

- Cytokine concentrations were generally higher after 48 h or 72 h of incubation than after 24 h (**Figure 4**), but not significantly so.
- No specimens for which a cytokine was undetectable after 24 h of incubation exhibited detectable cytokines after 48 or 72 h. In one case, cytokine was detectable after 24 h of incubation, but was undetectable after 72 h of incubation.
- It is unlikely that incubation for 72 h would lead to different conclusions about milk immune responses than 24 h, nor to observation of different patterns, and so we conclude that incubation beyond 24 h is not necessary.

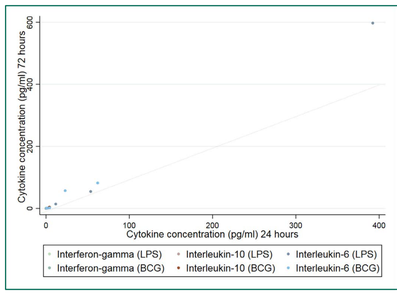


Figure 4. Cytokine concentrations estimated in specimens incubated with *M. bovis* BCG (BCG) or lipopolysaccharide (LPS) for **24 hours** (x) or **72 hours** (y). The diagonal line has a slope of one. Cytokine production was generally higher after 72 h, but not enough to change conclusions. Incubation beyond 24h is unnecessary.

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 8.70% 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**.

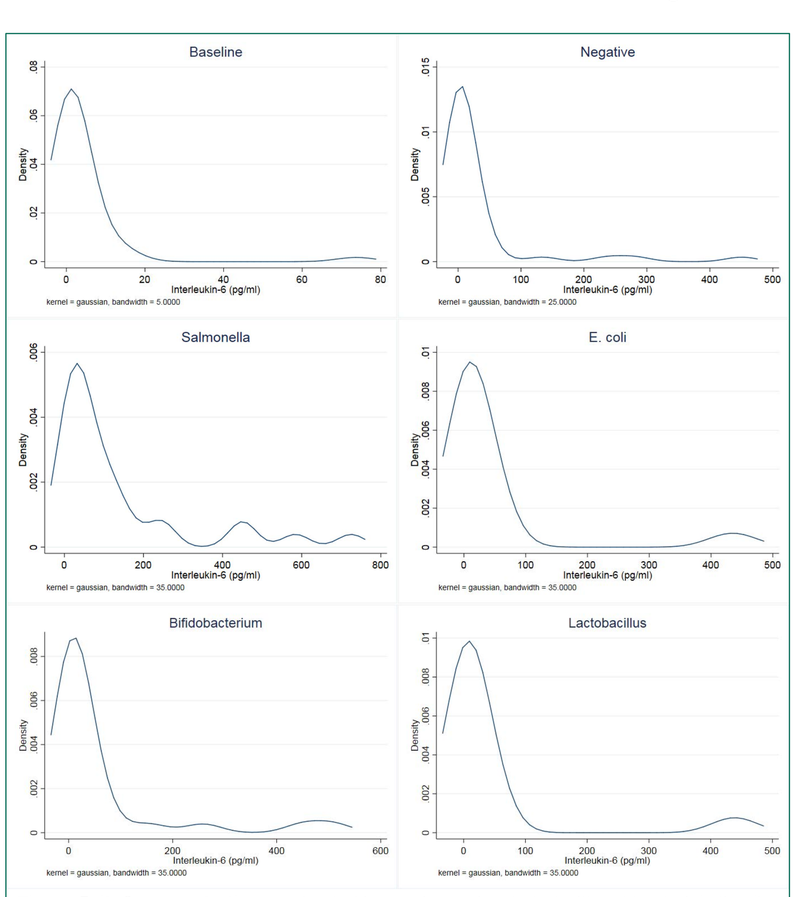


Figure 5. IL-6 concentrations estimated in specimens incubated with gut bacterial stimuli: **Baseline**, **Negative**, ***Salmonella***, ***E. coli***, ***Bifidobacterium***, ***Lactobacillus***. Undetectable levels of IL-6 were assigned the lower limit of detection of the assay. The distribution of IL-6 in milk at baseline is right-skewed, as are the distributions across all *in vitro* conditions.

Interleukin-6 responses to stimuli were characterized as ratios of stimulated IL-6 concentration to Baseline IL-6 concentration. These are shown in **Figure 6**.

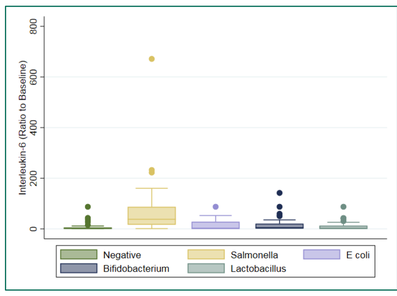


Figure 6. IL-6 response (ratio to Baseline) across all stimuli: **Baseline**, **Negative**, ***Salmonella***, ***E. coli***, ***Bifidobacterium***, ***Lactobacillus***. Like IL-6 concentrations, these ratios exhibit right-skewed distributions. IL-6 responses to the Negative condition were the smallest, and IL-6 responses to *Salmonella* were the largest.

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 largest.
- 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.

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References:

[1] Goldman AS. 2007. The immune system in human milk and the developing infant. *Breastfeed Med* 2.4.
[2] Cabinian A et al. 2016. Transfer of maternal immune cells by breastfeeding: maternal cytotoxic T lymphocytes present in breast milk localize in the Peyer's patches of the nursed infant. *PloS One* 11.6: e0156762.
[3] Ghosh MK et al. 2016 Maternal milk T-cells drive development of transgenerational Th1 immunity in offspring thymus. *J Immunol* 197.6.