Human milk cytokine responses to gastrointestinal bacteria measured via *in vitro* stimulation

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Introduction

Human milk contains numerous immune factors, which together constitute the "immune system of milk" [1]:

- White blood cells
- Antibodies (secretory immunoglobulin A)
- Immune cell communication molecules (cytokines)
- Antimicrobial factors (e.g., lactoferrin, fibronectin)
- Commensal microbes

The immune system of milk protects infants against infectious disease and guides their immune system development. Immune cells in milk can enter lymph tissue (Peyer's patches) in the infant gut to coordinate immune responses [2]. Maternal immune cells "train" infant immune cells, transferring lasting memory [3].

We have developed a technique to describe immune responses in whole human milk that is:

- interpretable at the *system* level (the immune system of milk)
- practical for population-based, international research

In this technique, milk specimens are combined with pathogenic or commensal gut bacteria, incubated at 37°C for 24 hours, and evaluated for cytokines. Comparison of cytokine concentrations in stimulated and baseline specimens provides a measure of immune cell activity—the immune response in milk.

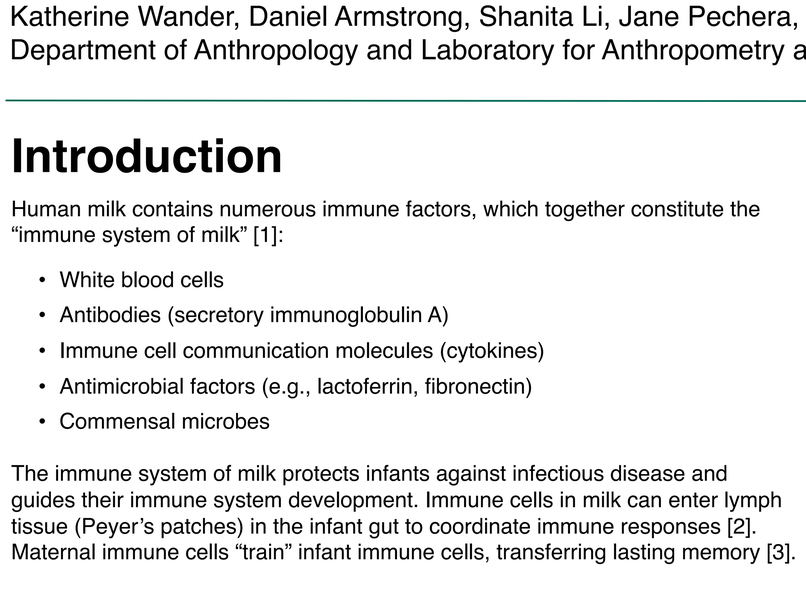

Figure 1. IL-6 concentrations (Panel A), and ratios to **Baseline** for IL-6 (Panel B), IL-10 (Panel C), and IFN-γ (Panel D) across conditions: **Negative** (unstimulated), ***Salmonella***, ***E. coli***, ***Bifidobacterium***, and ***Lactobacillus***. IL-6 responses to *Salmonella* were larger (all $p < 0.05$).

Methods

Participating women provided milk specimens by expression with an electric breast pump and provided information about their child, health, and breastfeeding practices.

One milliliter of milk was separated by centrifugation and the aqueous portion was frozen as a "Baseline" specimen. Within four hours of expression, two milliliters of milk were diluted with 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)] and a bacterial stimulus in a 6-well suspension culture plate in the following conditions:

- Unstimulated
- *Salmonella enterica* (Microbiologics 0363L)
- *Escherichia coli* (Microbiologics 0791E3)
- *Lactobacillus acidophilus* (Thermo Scientific Culti-Loops R4603050)
- *Bifidobacterium breve* (Thermo Scientific Culti-Loops R4606801)

Plates were placed in a glass desiccator in an anaerobic environment, created by burning a candle within the sealed desiccator (eliminating the need for use of pressurized CO_2). The desiccator was placed in an incubator at 37°C for 24 h.

The aqueous portion of baseline and incubated specimens was isolated by centrifugation and evaluated by multiplex enzyme immunoassay (QuanSys Biosciences, high-sensitivity human cytokine 4-plex assay) for the 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), and interleukin-10 (IL-10; an anti-inflammatory cytokine).

Milk fat was estimated with the creamatocrit method [4]: ~70 µl was drawn into a glass capillary tube and separated by centrifugation. Milk fat percentage was calculated from the height of the fat and total column.

Findings

In vitro stimulation with gut bacteria was completed for 29 participants.

Interleukin-6, a pro-inflammatory cytokine, was detectable in: 5 (17.24%) Baseline specimens, 25 (86.21%) specimens stimulated with *Salmonella*, 14 (48.28%) specimens stimulated with *E. coli*, 18 (62.07%) specimens stimulated with *Bifidobacterium*, and 11 (of 28; 39.29%) specimens stimulated with *Lactobacillus*.

Interleukin-10, and anti-inflammatory cytokine, was detectable in: Baseline: 2 (6.90%); *Salmonella*: 3 (10.34%); *E. coli*: 2 (6.90%); *Bifidobacterium*: 2 (6.90%); and *Lactobacillus*: 3 (of 28; 10.71%).

Interferon-γ, a mediator of innate and type 1 cell-mediated immunity, was detectable in: Baseline: 5 (17.24%); *Salmonella*: 7 (24.14%); *E. coli*: 6 (20.69%); *Bifidobacterium*: 5 (17.24%); and *Lactobacillus*: 5 (of 28; 17.86%).

Responses to gut bacterial stimuli: We used the assay's lower limit of detection to characterize undetectably low specimens and calculated ratios of stimulated to Baseline cytokine to characterize milk immune response to each stimulus.

Figure 1 shows IL-6 concentrations and IL-6, IL-10, and IFN-γ ratios to Baseline for all stimuli. IL-6 responses were most commonly observed.

IL-6 ratios to Baseline (geometric mean, range):

Salmonella: 26.9 (1, 671.4) *E. coli*: 3.88 (1, 87.4)
Bifidobacterium: 5.26 (1, 142.0) *Lactobacillus*: 2.87 (1, 87.4)

IL-10 ratios to Baseline (arithmetic mean, range):

Salmonella: 1.51 (1, 386.5) *E. coli*: 1.29 (1, 260.7)
Bifidobacterium: 1.35 (1, 275.3) *Lactobacillus*: 1.31 (1, 306.2)

IFN-γ ratios to Baseline (arithmetic mean, range):

Salmonella: 1.68 (0.05, 2,599.6) *E. coli*: 1.34 (0.05, 628.6)
Bifidobacterium: 1.18 (0.05, 663.8) *Lactobacillus*: 1.06 (0.01, 632.5)

Interleukin-6, IL-10, and IFN-γ responses (ratios to Baseline) were greater in the *Salmonella* condition. This was significant for IL-6 (all $p < 0.05$).

Table 1. Correlations across conditions: Interleukin-6 (ratio to Baseline)

	Salmonella	*E. coli*	*Bifidobacterium*	*Lactobacillus*
Salmonella	1	0.23 (0.2287)	0.25 (0.2001)	0.16 (0.4121)
E. coli	0.67 (0.0001)	1	0.68 (0.0000)	0.87 (0.0000)
Bifidobacterium	0.69 (0.0001)	0.82 (0.0000)	1	0.75 (0.0000)
Lactobacillus	0.55 (0.0024)	0.78 (0.0000)	0.74 (0.0000)	1

Pearson's ρ (p-value) in red; Spearman's ρ (p-value) in blue. IL-6 responses to *Salmonella* were not as strongly correlated with IL-6 responses to *E. coli, Bifidobacterium,* or *Lactobacillus* as responses to these stimuli were correlated with each other.

Associations across bacteria: The correlation matrix for IL-6 responses (**Table 1**) shows that correlations between IL-6 responses to *Salmonella* and *E. coli*, *Bifidobacterium*, or *Lactobacillus* were of smaller magnitude (Spearman's) and lower significance (Pearson's) than correlations between these three.

This suggests the immune system of milk responds differently to normal constituents of the gut than to the pathogenic bacterium, *Salmonella enterica*.

Associations across cytokines: Milk *in vitro* IL-6, IL-10, and IFN-γ responses to each bacterial stimulus were generally positively correlated (**Figure 2**).

The exceptions were IL-6 responses to *Salmonella*, which were unassociated with IL-10 (Spearman's ρ: 0.03; p: 0.8621) and IFN-γ (ρ: 0.30; p: 0.1116) responses to *Salmonella*. Dramatic IL-6—but not IL-10 or IFN-γ—responses to *Salmonella* were apparent (yellow lines in panels A and B).

It is notable that the specimens that exhibited the largest IL-10 or IFN-γ responses exhibited moderate IL-6 responses.

IL-6 and IL-10 responses were otherwise generally positively correlated:

E. coli: Spearman ρ: 0.42 (p: 0.0239)
Bifidobacterium: Spearman ρ: 0.31 (p: 0.1063)
Lactobacillus: Spearman ρ: 0.49 (p: 0.0084)

IL-6 and IFN-γ responses were also generally positively correlated:

E. coli: Spearman ρ: 0.34 (p: 0.0716)
Bifidobacterium: Spearman ρ: 0.08 (p: 0.6649)
Lactobacillus: Spearman ρ: 0.59 (p: 0.0009)

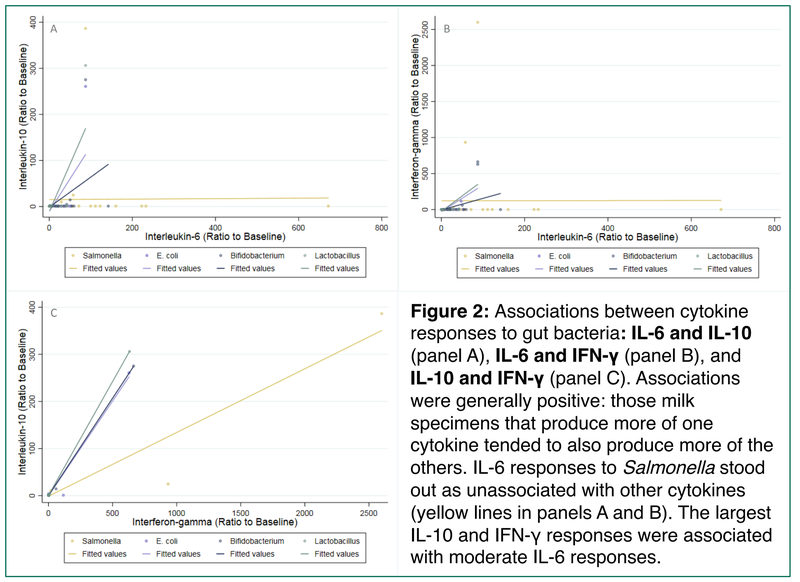

Figure 2: Associations between cytokine responses to gut bacteria**: IL-6 and IL-10** (panel A), **IL-6 and IFN-γ** (panel B), and **IL-10 and IFN-γ** (panel C). Associations were generally positive: those milk specimens that produce more of one cytokine tended to also produce more of the others. IL-6 responses to *Salmonella* stood out as unassociated with other cytokines (yellow lines in panels A and B). The largest IL-10 and IFN-γ responses were associated with moderate IL-6 responses.

Baseline predictors of cytokine responses: Higher **Baseline IL-6** was generally positively associated with all three cytokine responses (**Figure 3**). Baseline IL-6 was positively correlated with IL-6 response to *E. coli* (Spearman's ρ: 0.35, p: 0.0613), *Bifidobacterium* (ρ: 0.31; p: 0.1071), and *Lactobacillus* (ρ: 0.49; p: 0.0083), but not *Salmonella* (ρ: -0.07; p: 0.7166).

Baseline IL-6 was positively correlated with IFN-γ response to *Salmonella* (Spearman's ρ: 0.49; p: 0.0065), *Bifidobacterium* (ρ: 0.39; p: 0.0323), and *Lactobacillus* (ρ: 0.40; p: 0.0345), but not *E. coli* (ρ: 0.10; p: 0.6053). Baseline IL-6 was also positively associated with IL-10 responses.

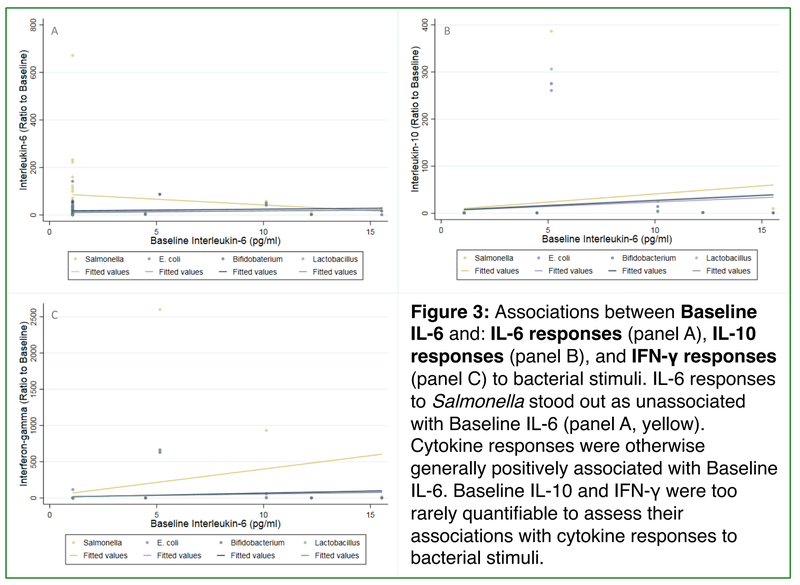

Figure 3: Associations between **Baseline IL-6** and: **IL-6 responses** (panel A), **IL-10 responses** (panel B), and **IFN-γ responses** (panel C) to bacterial stimuli. IL-6 responses to *Salmonella* stood out as unassociated with Baseline IL-6 (panel A, yellow). Cytokine responses were otherwise generally positively associated with Baseline IL-6. Baseline IL-10 and IFN-γ were too rarely quantifiable to assess their associations with cytokine responses to bacterial stimuli.

Baseline milk fat was not significantly correlated with cytokine responses to any bacterial stimuli (**Figure 4**).

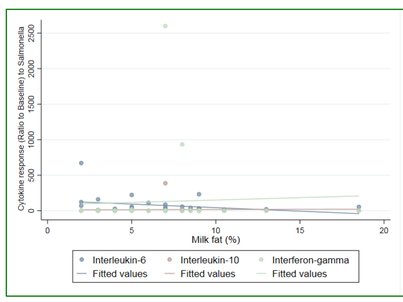

Figure 4: Associations between baseline **Milk fat** (%) was uncorrelated with **IL-6** (blue), **IL-10** (red), and **IFN-γ** (green) responses to *Salmonella.* Responses to other bacterial stimuli were similarly unassociated with milk fat (%).

Conclusions: The immune system of milk generally mounts pro-inflammatory responses *in vitro.* Increases in the pro-inflammatory cytokine IL-6 were more common that increases in the anti-inflammatory cytokine IL-10 and the type 1 cytokine IFN-γ.

IL- 6 responses to *Salmonella* stood out in potentially important ways: They were larger in magnitude (Figure 1) than responses to other bacteria; they were unassociated with IL-6 responses to other bacteria (Table 1); and, they were unassociated with other cytokines responses to *Salmonella* (Figure 2).

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References:

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