Predictors of human milk immune responses to gastrointestinal bacteria

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Introduction

Human milk benefits maternal and child health through multiple immune factors. Antibodies, cytokines, white blood cells, and antimicrobial factors protect infants against infection and influence immune system development [1]. We have developed a protocol to describe milk *in vitro* immune responses for use across a range of field conditions to support further investigation of the immune system of milk.

We used this protocol to describe milk *in vitro* immune responses to gastrointestinal bacteria among women in upstate New York. Here, we evaluate predictors of pro-inflammatory responses to *Salmonella enterica*, a gastrointestinal pathogen. We predicted stronger pro-inflammatory responses associated with:

- infectious disease symptoms in mothers or children [2]
- maternal immune-mediated disease (allergy or autoimmunity)
- maternal investment [3]

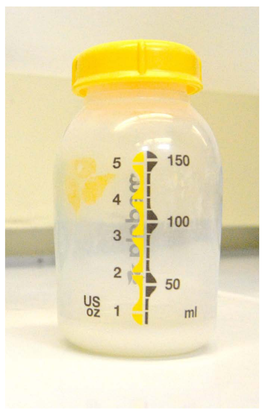
Table 1. Sample characteristics (N = 29)

Twin births	1	3%
Mother's self-reported race:		
Asian	2	6.9%
Hispanic	2	6.9%
Mixed	1	3.45%
White	24	82.76%
Mother born outside of the US	1	3.45%
Child receiving any complementary food	20	68.97%
Mother ill at the time of data collection*	7	24.14%
Mother has an autoimmune disease**	5	17.24%
Mother has an allergic disease**	3	10.34%
Child ill at the time of data collection*	6	20.69%
Maternal age in years (mean; range)	29.3	21-40
Child age in months (mean; range)	9.1	1-26
Child birthweight in grams (mean; range)	3445	2268-4365
Interbirth interval with older sibling in months (mean; range)	42.7	21-93

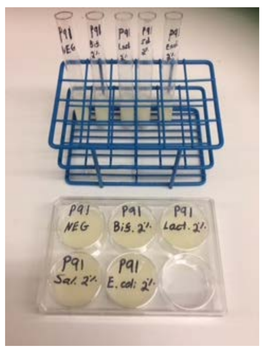
*Reported symptoms were generally mild. None had been diagnosed with an infectious disease in the preceding week. **Physician-diagnosed disease (as reported by participants).

Methods

Forty-nine breastfeeding women expressed milk via electric pump and provided information regarding themselves (age, country of birth, physician-diagnoed allergy or autoimmune disease, and symptoms of infectious disease within the preceding week) and their breastfeeding child(ren) (age, birth weight, birth order, interbirth interval from older sibling, and symptoms of infectious disease the preceding week).



Milk was diluted in 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)] containing a bacterial stimulant (2 ml milk:1 ml of medium) and incubated in an anaerobic environment at 37°C for ~24 hours.



The aqueous portion of baseline and incubated specimens was isolated and evaluated for cytokines via multiplex enzyme immunoassay.

Four bacterial stimuli were considered (all Thermo-Fisher QuantiCult or QuantiLoops), as well as a Negative (unstimulated) control:

- *Bifidobacterium breve* (Bif)
- *Lactobacillus acidophilus* (Lact)
- *Salmonella enterica* (Sal)
- *Escherichia coli* (E. coli)

Four cytokines were considered:

- Interleukin-4 (IL-4; a type-2 cytokine)
- Interleukin-6 (IL-6; a pro-inflammatory cytokine)
- Interleukin-10 (IL-10; an anti-inflammatory cytokine)
- Interferon-γ (IFN-γ; a type-1 cytokine)

Findings

Interleukin-6 responses to *Salmonella* were evaluated among 29 participants (**Table 1**). IL-6 was most frequently quantifiable in stimulated specimens. IL-6 responses to stimuli were quantified as the ratio of the concentrations in the stimulated and baseline specimens.

Salmonella was the most immunogenic (geometric mean ratio to Baseline: 26.9, range: 1.0-671.4). We evaluated associations between *Salmonella*:Baseline IL-6 and maternal and child characteristics (**Figure 1**).

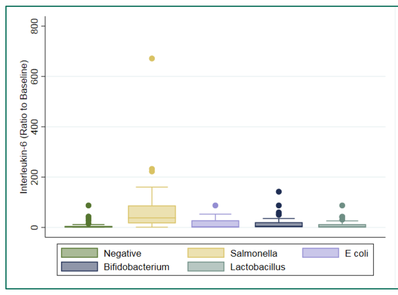


Figure 1. Interleukin-6 (Ratio to Baseline) responses to **Negative control**, ***Salmonella***, ***E. coli***, ***Bifidobacterium***, and ***Lactobacillus*** conditions. IL-6 responses were consistently highest for the *Salmonella* condition.

Maternal Characteristics were evaluated for univariate associations with IL-6 responses to *Salmonella.*

We predicted increased maternal investment in the immune system of milk among older mothers (reflecting lower cost to investment in current reproduction); however, **maternal age** (mean:29.3, range:21-40) was unassociated with IL-6 responses to *Salmonella* (Spearman's p:0.2839; Pearson's p:08098; **Figure 2**)

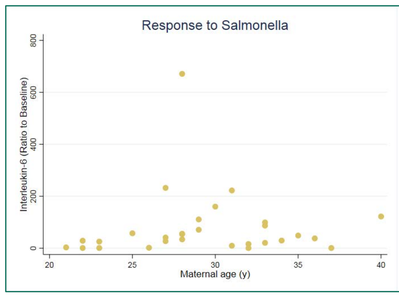


Figure 2. Maternal age and interleukin-6 response to *Salmonella* (ratio to Baseline). No association was apparent.

Physician-diagnosed maternal allergies were reported by 3 participants (10.34%). IL-6 responses to *Salmonella* were not discernably different (**Figure 3**) among these participants. IL-6 responses to the Negative control (no bacterial stimulus) condition were also no discernably different among these participants.

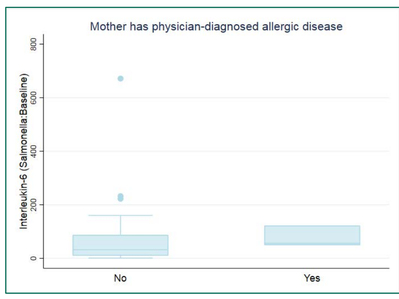


Figure 3. Maternal allergy (physician-diagnosed) and interleukin-6 response to *Salmonella* (ratio to Baseline). No association was apparent.

Seven mothers (24.14%) reported feeling **symptoms of illness** at the time of participation. These included respiratory symptoms (coughing, sneezing, sore throat, runny nose), headache, and mastitis (one participant). There was no significant difference between ill mothers and healthy mothers (*p*: 0.2372) (**Figure 4**). This was inconsistent with our prediction that maternal infectious disease would produce stronger milk immune responses.

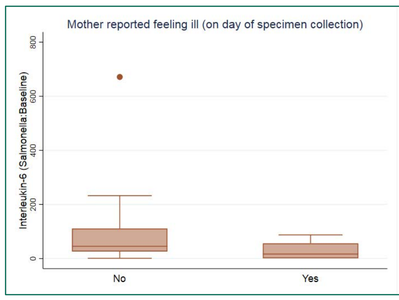


Figure 4. Maternal symptoms of infectious disease and interleukin-6 response to *Salmonella* (ratio to Baseline). No association was apparent. Notably, reported symptoms of illness were mild; none of the participating mothers was ill enough to have been diagnosed by a physician with an infectious disease within the preceding week.

17.24% of participants reported a **physician-diagnosed autoimmune disease**. Hashimoto's or postpartum thyroiditis were the only condition reported among the women evaluated for IL-6 responses to *Salmonella*; in the larger study sample (N: 49), coeliac disease, multiple sclerosis, and fibromyalgia were also reported. IL-6 responses to *Salmonella* were not significantly different among these women (*p*: 0.8083); IL-6 responses to the Negative condition were significantly higher (*p*: 0.0220; N: 49) (**Figure 5**). This suggests that the immune system of milk in autoimmune mothers is more likely to mount inappropriate responses.

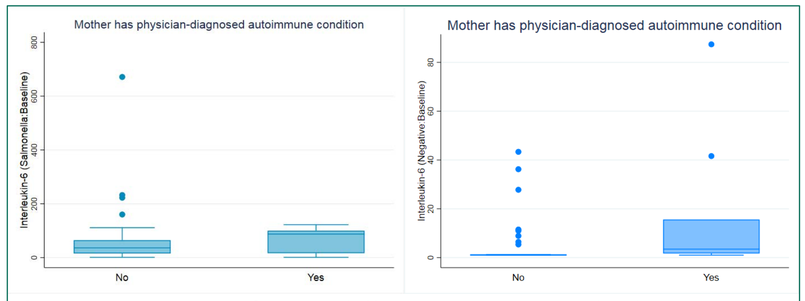


Figure 5: IL-6 responses to *Salmonella* (left) and the Negative control (right) among mothers with and without a **physician-diagnosed autoimmune condition.** Autoimmune disease was associated with significantly higher IL-6 responses to the Negative control, suggesting milk from autoimmune mothers may be more likely to mount inappropriate responses.

Child Characteristics were evaluated for univariate associations with IL-6 responses to Salmonella.

There was no correlation between child age and IL-6 response to *Salmonella* (*p*: 0.5527; **Figure 6**).

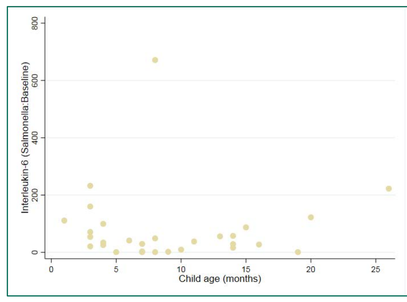


Figure 6. IL-6 response to *Salmonella* and **child age** (in months). There was no significant correlation.

We predicted higher maternal investment in higher birthweight (bw) children to be reflected in milk immune responses; however, there was no correlation between **child bw** and milk IL-6 response to *Salmonella* (**Figure 7**). We also predicted higher maternal investment in higher birth order children (as with maternal age, reflecting lower costs to investing in current reproduction); however, there was no correlation between **birth order** and IL-6 response to *Salmonella* (**Figure 7**).

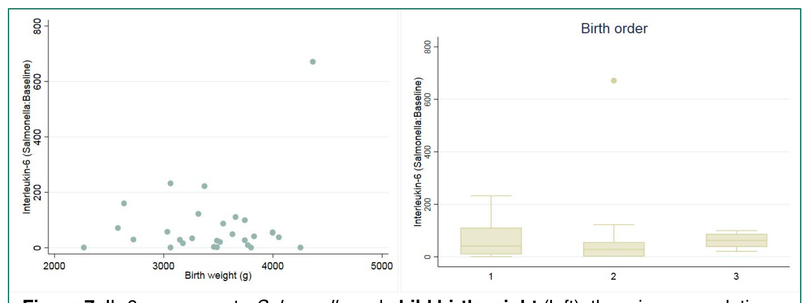


Figure 7. IL-6 responses to *Salmonella* and **child birthweight** (left); there is no correlation. IL-6 responses to *Salmonella* by **birth order** (right); again, there is no significant association.

Six mothers (20.69%) reported observing **signs of illness in the breastfeeding child** on the day of participation. We predicted that the immune system of milk would respond to such child illness with elevated immune responses; however, we observed no significant association (Spearman's *p*: 0.4121) between IL-6 responses to *Salmonella* and signs of child illness (**Figure 8**).

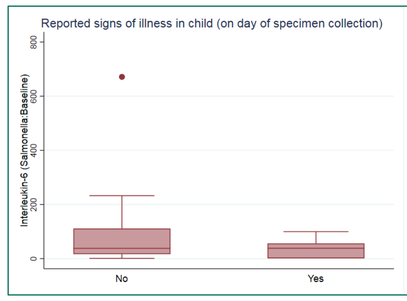


Figure 8. Reported signs of illness in the breastfeeding child and IL-6 responses to *Salmonella* (ratio to Baseline). No significant difference between milk produced for children who were ill and those who were not was apparent. Notably, reported signs of illness in children were mild; none was ill enough to have been diagnosed by a physician with an infectious disease within the preceding week.

Conclusions: IL-6 responses to *Salmonella* were highly variable, and greater in magnitude than responses to other gastrointestinal bacteria. They did not exhibit patterns consistent with higher maternal investment in milk for high birthweight children, nor higher maternal investment at the end of the reproductive career. We observed higher IL-6 responses to the Negative condition among mothers with diagnosed autoimmune disease, suggesting that milk immune responses may reflected maternal immune pathology, which bears further investigation.

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References:

[1] Goldman AS. 2007. *Breastfeed Med* 2(4):195-204.
[2] Breakey AA et al. 2015. *Evol Med Pub Health* 2015(1):21-31.
[3] Wander K, Mattison SM. 2013. *Proc R Soc B* 280(1768):20131359.