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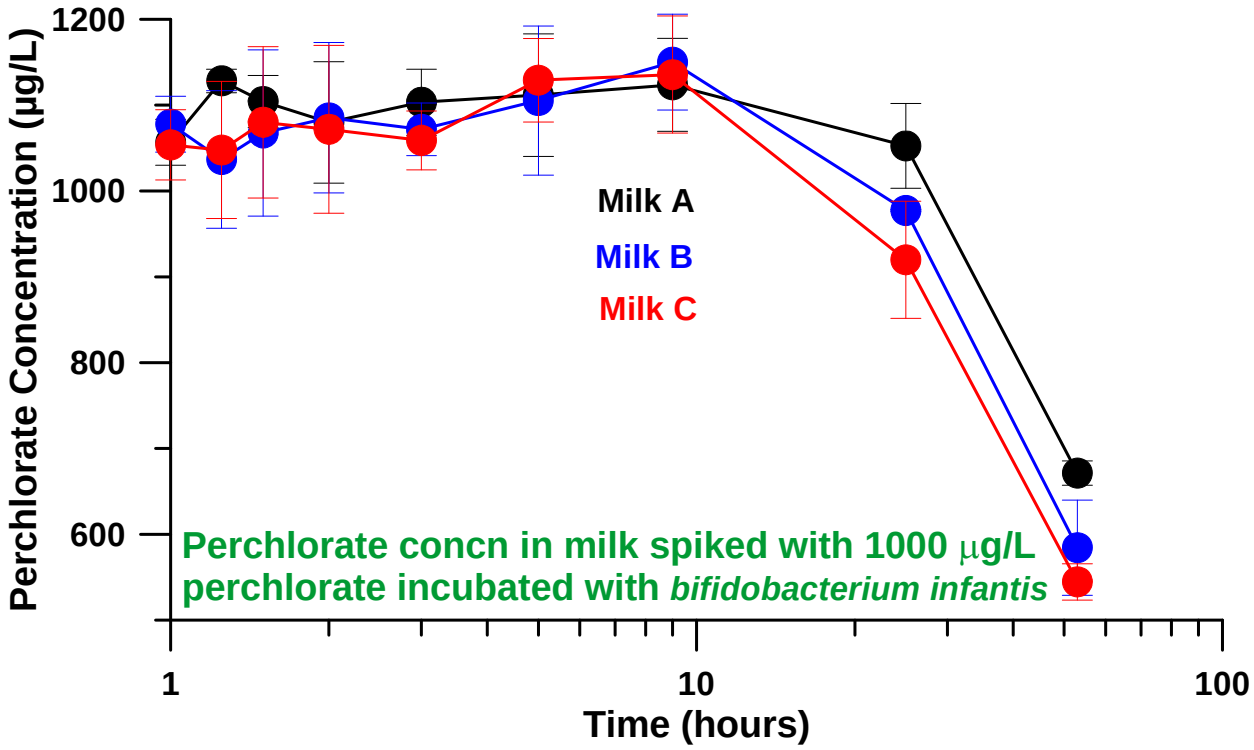
Table of Content Graphic



Breastfed Infants Metabolize Perchlorate

C. Phillip Shelor,[†] Andrea B. Kirk,[†] Purnendu K. Dasgupta,[*,†] Martina Kroll,[†] Catrina A. Campbell,[†] Pankaj K. Choudhary[‡]

[†]Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX 76019-0065, USA

[‡]Department of Mathematical Sciences, University of Texas at Dallas, 800 W. Campbell Rd., Richardson, TX 75080-3021

ABSTRACT

Bifidobacteria are the dominant intestinal bacteria in breastfed infants. It is known that they can reduce nitrate. Although no direct experiments have been conducted until now, inferred pathways for *Bifidobacterium bifidum* include perchlorate reduction via *perchlorate reductase*. We show that when commercially available strains of *bifidobacteria* are cultured in milk, spiked with perchlorate, perchlorate is consumed. We studied 13 breastfed infant-mother pairs who provided 43 milk samples and 39 infant urine samples, and 5 formula-fed infant-mother pairs who provided 21 formula samples and 21 infant urine samples. Using iodine as a conservative tracer, we determined the average urinary iodine (UI) to milk iodine (MI) concentration ratio to be 2.87 for the breastfed infants. For the same samples, the corresponding perchlorate concentration ratio was 1.37 (difference significant, $p<0.001$), indicating that perchlorate is lost. For the formula fed infant group the same ratios were 1.20 and 1.58; the difference was not significant ($p = 0.68$). However, the small number of subjects in the latter group makes it more difficult to conclude definitively whether perchlorate reduction does or does not occur.

Keywords: Perchlorate, Infant Iodine Nutrition, Microbial Reduction, *Bifidobacteria*

[*]Corresponding Author. Email Address: Dasgupta@uta.edu

INTRODUCTION

Perchlorate is a common contaminant of food and drinking water.[1] The toxicity of perchlorate and its regulation have been the focus of considerable controversy in the US.[2-5] Perchlorate is an endocrine disruptor. Adequate iodine uptake, especially in infants, is critical. Perchlorate competitively inhibits uptake of iodine into the thyroid gland, potentially reducing production of thyroid hormones, thyroxine (T4) and tri-iodothyronine (T3). It thus acts as a potential neurotoxicant as these hormones are essential for neurodevelopment.[6] The sodium iodide symporter (NIS) is a glycosylated 90-97 kDa protein that is responsible for shuttling iodide to the thyroid for the biosynthesis of the thyroid hormones. Perchlorate inhibition of iodide transport arises because the NIS actually has greater affinity for perchlorate than it does for iodide.[7] The NIS is well-expressed in the mammary glands during lactation and serves to transport essential iodine to the young.

While in hindsight, the presence of perchlorate in milk is not surprising, our original report of perchlorate in bovine[8] and later human milk[9] generated much skepticism. Determination of perchlorate in complex matrices may have been initially challenging to us,[10-15] but chromatography-tandem mass spectrometry has become more affordable and sensitive over the intervening period and is now routine. Perchlorate has been used as an ingredient in solid fuel rocket propellants, explosives and fireworks. It also occurs naturally, appearing in Chilean mineral deposits (Chilean nitrate has been widely used as fertilizer), rain water, and some soils and groundwater. The origin of natural perchlorate was also of interest.[16,17]

We noted that perchlorate excretion in human milk can be highly variable between subjects and in the same subject at different times.[18] Relative to either iodide or thiocyanate, a greater fraction of perchlorate was present in milk compared to the ratio

of the same species in the urine of lactating mothers.[19] Concerns about perchlorate aside, milk iodine concentrations (MI) of many mothers were very low. Iodine nutrition of infants of such mothers would be in jeopardy regardless of the concurrent presence of perchlorate.

Perchlorate at low levels is pervasive in our environment. Remedial measures are not likely to remove all or even the major sources of perchlorate anytime soon. Given limited resources, if a choice is to be made between *more iodine or less perchlorate*[20] to improve iodine nutrition, one of us concluded[21] that we should concentrate on the former. The same sentiment was echoed later in a report by the EPA Office of the Inspector General;[22] this may not, however, be a universally shared opinion, even among the present authors.

Present wisdom holds that ingested perchlorate is not metabolized in humans[4,23] and passes through with a relatively short clearance time.[24-26] Perchlorate is known to be reduced in the rumen of ruminant animals,[27] although plants are more practical bioremediation means than cows. While perchlorate is translocated and not really reduced in lettuce,[28] other plant systems, e.g., Poplar trees, do reduce perchlorate.[29] Even with plants, microbially mediated rhizodegradation is likely more important than phytodegradation.[30-33] A considerable variety of bacteria can reduce chlorate and perchlorate (often denoted as (per)chlorate) as well as nitrate, given suitable oxidizable substrates (this includes a great variety of organic anions).[34]

Despite conventional wisdom, fermentation similar to that occurring in the rumen is known to occur in the large intestine.[35] Facultative anaerobes are essential intestinal flora in both infants and adults. Nature has been making perchlorate for eons.[36] Natural perchlorate has thus appeared at significant concentrations in some regions in water used by humans for time immemorial, in much the same way as it does

today.[37] In Chilean cities near the Atacama Desert where perchlorate content in water and in mother's milk is relatively high, no unusual neonatal thyroid problems have been reported.[37,38] Some of the effects of perchlorate in such populations may be ameliorated by co-occuring high iodine levels; however, one cannot help but wonder if that is the sole reason that these infants can handle breast milk perchlorate concentrations (MPC) in excess of 100 μg/L without obvious effect on thyroid development.

In this paper we first show that *bifidobacteria,* facultative anaerobes that constitute the dominant bacteria in the digestive system of breastfed infants, can reduce perchlorate in a milk matrix *in-vitro*. Metabolization of perchlorate[27] by cattle was proven by deliberately feeding significant concentrations of perchlorate to the animals and following its concentration in various body fluids/excreta. Given the known effects of perchlorate, such an experiment cannot be ethically conducted with human infants. We rationalize why iodine can be used as a conservative tracer and thus devise a means to determine if any of the perchlorate ingested by infants through breastmilk or formula is metabolized. The evidence indicates that perchlorate must be significantly metabolized in breastfed infants.

■ EXPERIMENTAL SECTION

Bacterial Cultures and analysis for Perchlorate. All bacterial experiments were conducted with store-bought Pasteurized skim milk purchased locally and except as stated, spiked to contain nominally 1 mg/L ClO_4^-. (A more limited set of experiments were conducted with milk samples spiked nominally with 100, 250, 500, and 5000 μg/L ClO_4^-.) Ten milliliter aliquots were pipetted into 15 mL screw-cap culture tubes. All experiments were conducted in triplicate; commercially available probiotic supplements, stated to contain live bacteria were added to the spiked skim milk. To

each tube was added the contents of one capsule of *Align*™ (*bifidobacterium infantis* 35624, Procter and Gamble, each capsule contains 4 mg of the bacterial mass, labeled to contain 10^9 colony forming units (cfu) when packaged, plus other material[39]). As a point of reference, the bifidobacterial count in the feces of one-month old breastfed infants has been reported to be 200 x 10^9 cfu/g.[40]

To another similar sample set was added a probiotic blend (Advanced Probiotic 10, Nature's Bounty, Inc.). This product is labeled to contain 10^{10} cfu's/capsule of a bacterial mixture consisting of *lactobacillus plantarum*, *bifibacterium bifidum*, *lactobacillus rhamnosus*, *lactobacillus bulgaricus*, *lactobacillus salivarius*, *lactobacillus brevis*, *lactobacillus acidophilus*, *bifidobacterium lactis*, *lactobacillus paracasei*, and *lactobacillus casei* in an unspecified ratio.

The tubes were capped and placed in a 37 °C bath. Over a period of 2 days, 50 µL sample aliquots were taken in triplicate of each sample at logarithmically spaced intervals (sampling was more frequent early in the culture process). The tube was vortexed for homogenization prior to sampling. The samples developed a sufficiently gelled growth at the bottom of the tubes after 24 h; homogenization of the samples by vortexing could not necessarily be assured. If the sample aliquot thus contained more of the free liquid portion, it would be biased towards containing more perchlorate (a soluble anion), rather than less perchlorate than the sample as a whole. Samples were pipetted into 1.7 mL capacity graduated polypropylene microcentrifuge tubes (www.midsci.com) and subjected to a modified Fenton digestion method.[41] To the sample in the microcentrifuge tube 50 µL of 150 µg/L $^{35}Cl^{18}O_4^-$ (as $NaClO_4$, 95% isotopic purity, www.iconisotopes.com), 200 µL 30% H_2O_2, and a small spatula tip (7±3 mg) of iron powder (P/N 00170, 99.9+% metals basis, <10 µm spheres, www.Alfa.com). Ferrous salts are typically used in conventional Fenton oxidation; the reaction is fast but results in incorporation of the anion associated with Fe(II). For small samples that are

not preferably diluted by large amounts, elemental iron provides an alternative; the reaction is slower but anion contamination is avoided.[41,42] The samples were digested in a 60 °C convection oven for 3 days to ensure complete digestion, which is slower because of the surface area and diffusion limitations of the iron powder. DI water was added to make up for water loss each day and to rinse the walls of the tube. After 3 days, 50 μL of 4 M KOH was added to precipitate any dissolved iron and a small spatula tip full of MnO_2 (2.4±0.8 mg) was added for the catalytic removal of unreacted H_2O_2. Samples were then diluted to 1.5 mL, centrifuged at 2000g, filtered through 0.2 μm nylon syringe filters into autosampler vials, capped, and analyzed by Ion Chromatography–tandem Mass Spectrometry (IC-MS/MS) with isotope ratio based quantitation (*vide infra*).

Human Subject Recruitment and Sample Collection. Our protocol, approved by the Institutional Review Board at the University of Texas at Arlington, called for the recruitment of mother-infant pairs where the infants are below 7 months of age and are either exclusively breastfed or exclusively formula fed (no water, juice, or solid foods). Breastfed infant ages ranged from 1.3-7.0 mo. old at the time of sampling with an average age of 3.5 ± 1.6 mo. Formula fed infants ranged from 4.3-6.2 mo. old with an average age of 5.5 ± 0.7 mo. The mother was to collect (a) at least three and up to five breastmilk or formula samples (≥ 5 mL), alternating between immediately before and immediately after feeding the infant (in the case of formula, collecting an aliquot from the same batch fed to the infant), (b) at least three and up to five infant urine samples (≥ 5 mL) that represented the first micturition ≥ 15 min after the above feeding, (c) a corresponding number of maternal urine samples (only for breastfeeding mothers) collected at a time proximate (before or after) the feeding; for some volunteer breastfeeding mothers, 24-h urine samples were also collected. The maternal urine data are not used in this paper and are not further discussed. The samples were stored

refrigerated by the mother until the requisite number was collected. Either the investigators picked up the sample or it was delivered to our laboratory on ice. Once in the laboratory, the samples were stored frozen at -20 °C until analysis, typically in a few weeks. The only exclusion criteria was known thyroid disease/impairment for either mother or infant and any recent medical procedure that involved the use of contrast media (which frequently contains iodine).

While infant urine collection bags are routinely used in a hospital setting, the successful use of such bags in a home setting by mothers proved far more difficult. Many mothers found it frustrating that leakage occurred or an adequate volume was not collected, compounded by the obvious displeasure of the subjects, especially when the bag was fastened tightly to prevent leakage. Primarily for this reason, the dropout rate after recruitment was nearly 50%. We had thirteen breastfeeding mother-infant pairs and five formula fed infants successfully complete the study; two of the latter were fed soy-based formula.

Iodinalysis. Recently we developed a new digestion procedure based on the Fenton reaction.[41] The method was validated in comparison to a method used by the FDA that uses perchloric acid digestion and Sandell-Kolthoff colorimetry. Several of the breast milk (that were submitted with sufficient volume) and the formula samples from this study were in fact part of the above intercomparison.[41]

Briefly, to 0.5 mL of a milk or formula sample pipetted into a 15 mL screw-cap polypropylene centrifuge tube, 50 μL of 1 μg/mL ^{129}I tracer (carrier-free KI, www.ipl.isotopeproducts.com) was added. 0.5 mL of 30% H_2O_2, followed by 50 μL of 0.1 M $Fe(NH_4)_2(SO_4)_2$ were then added. After ~15 min, 25 μL of 2% NH_4OH was added. The centrifuge tube was loosely capped but not screwed shut. Fenton digestion was carried out in a convection oven overnight (12+ h) at 60 °C. After allowing the digest to

cool to room temperature, the digest was diluted to 5 mL with 2% NH_4OH to precipitate $Fe(OH)_3$. The tubes were then vortexed and centrifuged (3000g at 2°C, 15 min). The supernatant was directly subjected to Induction Coupled Plasma Mass Spectrometry (ICP-MS) analysis as detailed below. Urine samples were simply diluted prior to ICP-MS analysis, typically by 10x. Some required further dilution after the initial analysis to be within calibration range.

An X Series II ICP-MS (www.thermo.com) was used in the direct infusion mode. The peristaltic pump built into the ICP-MS was used to prime the sample into the Peltier-cooled (3 °C) nebulizer at 1.6 mL/min for 45 s and then continuously aspirate the sample into the nebulizer at 0.8 mL/min. Each measurement cycle consisted of a 20-s qualitative mass survey scan followed by three 32-s long quantitative mass scans. Between samples, 1% NH_4OH was used for a monitored washout up to 4 min to eliminate effects of any carryover between samples. After analysis was complete, the autosampler probe was washed in the same wash solution before storage and reuse.

Determination of Perchlorate. For urine samples, 2.5 mL of the sample was spiked with 12.5 µL of 1 mg/L $^{35}Cl^{18}O_4$ as the internal standard. Strong acid type H^+-form cation exchanger macroreticular resin (Dowex HCR-W2) was washed sequentially with methanol and water. Excess moisture was purged with air, without drying the resin. An aliquot of the resin (~250 mg) was added to the urine sample, vortexed, allowed to stand for 15 min, filtered through a 0.20 µm syringe filter into a sample vial and capped and loaded onto the autosampler.

To 5 mL of formula or milk (if sample availability was limited, the sample was diluted beforehand and the diluted sample used), 25 µL 1 mg/L $^{35}Cl^{18}O_4^-$ was added and the mixture was then centrifuged (3000g, 15 min, -2 °C). The casein, whey, and fat separated. 2.5 mL of the whey (avoiding the fat and the casein) was pipetted into a 10 kDa centrifugal filter (Vivaspin 6, www.sartorius-stedim.com). The whey was then

centrifuged (10000g, 90 min, 2 °C on a 25° fixed angle rotor). To the filtrate, approximately the same mass fraction of the aforementioned cation exchange resin was added (~100 mg /mL filtrate). The sample was then vortexed, allowed to stand for 15 min, filtered through a 0.20 μm syringe filter into a sample vial and capped and loaded onto the autosampler.

The IC-MS/MS analysis protocol used an IC-25 isocratic pump with an EG40 electrodialytic eluent generator, 2 mm bore AG21/AS21 guard and separation column sets housed in a LC30 temperature controlled oven (30 °C), ASRS-Ultra II anion suppressor in external water mode, and a CD-25 conductivity detector, all from ThermoFisher/Dionex. Around the elution window of perchlorate (t_R ~9 min), 6-10 min after injection, a diverter valve directed the CD25 effluent to a tandem mass spectrometer (Thermo Scientific Quantum Discovery Max with a heated ESI probe and enhanced mass resolution). Eletrodialytically generated high purity KOH eluent was used isocratically at a concentration of 15 mM and a flow rate of 0.35 mL/min. Eluent generation, sample injection (2 μL), electrodialytic suppression, autoranging conductivity detection and data acquisition were all conducted under Excalibur/Chromeleon software control. Perchlorate was quantified by the -99 → -83 Th (m/z) transition ratioed to the -107 → -89 Th internal standard transition. All data were interpreted in terms of a 5-point calibration with check standards run daily. Any sample falling outside the calibration range was reanalyzed after appropriate dilution. All samples were minimally analyzed in duplicate.

The limits of quantitation for iodine and perchlorate were 0.3 and 0.015 μg/L, similar to those reported previously.[19] Concentrations of both analytes in all of the analyzed samples were substantially above the limit of quantitation of the methods.

■ RESULTS AND DISCUSSION

Microbial Perchlorate Reduction. A number of perchlorate-reducing bacteria have been isolated and used in bioremediation of perchlorate-contaminated soils, sewage, and water systems.[43] Reduction of (per)chlorate typically occurs under anaerobic conditions, and the released oxygen is then used for respiration. All living systems that reduce perchlorate do so with the help of an enzyme *perchlorate reductase,* the same enzyme also reduces chlorate and nitrate. In fact, all bacteria that reduce perchlorate preferentially reduce nitrate if both are available.[43-45] The enzyme *perchlorate reductase* is a member of the Type II DMSO family and is closely related to *nitrate reductase*. Enzymes in this family all use a common molybdenum cofactor, [bis(molybdopterin guanine dinucleotide)-molybdenum.[46,47] The dominant pathways for metabolism of perchlorate in plants and animals are both likely mediated by microorganisms.[30,31]

Bacterial populations present in the human intestine are diet-dependent. Populations and ratios vary over the course of a lifetime, and also among populations of humans.[48] There are numerous studies that show that the intestinal bacterial population is distinctly different for breast-fed vs. bottle-fed infants. In breastfed infants, *bifidobacteria* far outweigh other bacteria;[49-51] another important group of anaerobic bacteria, *Ruminococci,* are also present in breast-fed infants.[52,53] In bottle-fed infants the *bifidobacteria* concentrations are lower and can sometimes be below the culture threshold limit (10^4 cfu/mL) although detection by PCR methods is possible.[54] Even under conditions when bifidobacteria become the dominant intestinal bacteria in bottle-fed infants, their number count remains an order of magnitude lower than those in breastfed infants.[40] Interestingly, infant intestinal *bifidobacteria* counts are higher for infants of breastfeeding mothers with no allergies compared to those with documented allergy problems.[55] Bifidobacterial diversity has been suggested as an index of systemic immune responses.[56] *Bifidobacteria* are routinely sold as supplements[57] and there are

ongoing studies to devise additives to infant formula that will have a bifidogenic effect.[58]

It is well established that *bifidobacteria* can reduce nitrate[59,60] but no direct experiments have ever shown that *bifidobacteria* can reduce perchlorate. However, inferred pathways for the taxon *Bifidobacterium bifidum* and its descendants include perchlorate reduction via *perchlorate reductase*.[61] Below we describe the results of *in-vitro* experiments do determine whether *bifidobacteria* can reduce milk spiked with perchlorate.

Perchlorate Temporal Profile in Perchlorate-spiked Milk Inoculated with *Bifidobacteria*. The supplement preparations contain other insoluble (and soluble) material aside from the bacterial mass. Conceivably these may remove perchlorate by adsorption or ion exchange. A two-point measurement (before and some fixed point after inoculation) is therefore inadequate as any removal by the solids in the supplement will be unaccounted for. Therefore, time based measurements were carried out. The results are shown in Figure 1. It will be noted that there is really no statistically significant reduction of the perchlorate content over the first 8 h. (Note that relative to adding no inoculum, the standard deviation of replicate sample analysis always increases significantly after adding the inoculum, possibly due to the intrinsic variability of any biological process). A small but statistically significant reduction is seen after 24 h and an obviously large reduction occurs after 52 h of incubation. Unlike a real digestive system, we do not have a system at steady state; our experiments start out in a clearly oxic condition and doubtless contains other reducible substrates like nitrate that may be preferentially reduced. A similar lag period in microbial perchlorate reduction has been reported by others.[62] As was noted in the experimental section, significant part of the culture medium gels/solidifies after ~24 h and homogenization cannot be assured by vortexing. Any intrusive mechanical homogenization was

avoided to preclude contamination by other adventitious species that will compromise the utility of the subsequent samples. There is however, no significant volume increase associated with the yogurt formation that will create an analytical bias on volume based analysis. To preclude any possibility that perchlorate is somehow preconcentrated in the solid, we carried out analysis of the solid portion per unit mass basis for the 5 mg/L sample. No preferential concentration of perchlorate in the gel phase over the original concentration per unit mass was found. Perhaps most importantly, the other inoculum with the "Probiotic Blend", which we expected to behave in the same fashion, did not; rather it served as a useful control. Gelling/yogurt formation was observed after about the same length of time, but as Figure 1 shows, there was no reduction of perchlorate concentration in this case even after 52 h. Whether the specific species of *bifidobacteria* present in the blend are not as effective for perchlorate reduction or the absolute *bifidobacteria* content are not nearly as high, is not known.

Finally, we show the fraction of perchlorate lost after 24 h as a function of perchlorate concentration in Figure 2. Granted that the uncertainties are very high, the fraction of perchlorate reduced after 24 h monotonically increases with increasing $[ClO_4^-]$. The fraction of $[ClO_4^-]$ consumed should be proportional to $(K_c[ClO_4^-]/(K_R[R] + K_c[ClO_4^-])$ where [R] is the concentration of competing reducible substrate and the K-terms are a composite representation of the binding constant to the substrate and the rate constant for its reduction. It will be obvious that for $K_R[R] >> K_c[ClO_4^-]$, the value of the overall expression will essentially linearly increase with $[ClO_4^-]$. The solid line in Figure 2 represents a best fit line that fits the expression $(a/(1+b/[ClO_4^-])$ where a and b are constants, concordant to the expression given above in terms of [R] and $[ClO_4^-]$ at constant [R].

It should not be construed that the precise dependence of the extent of perchlorate reduction on the absolute concentration observed in these in-vitro

experiments is quantitatively extrapolatable to infants. Neither the complete composition of the microflora, nor the precise redox conditions in the infant intestine are the same as that in a culture tube. In a real digestive system there are very likely other agents that metabolize competing oxidants like nitrate, without *bifidobacteria* derived *perchlorate reductase* pathway being the only agent for removal of such oxidants. In addition, the digestive system already contains a very large number of *bifidobacteria* at steady state (fecal count in a month –old breast fed infant is 2×10^{11} cfu/g,[40] compared to 10^8 cfu/mL at the beginning of our incubation); we therefore expect perchlorate reduction to be more efficient *in-vivo.*

Does *Bifidobacteria* Reduce Perchlorate in Infants? Since deliberately feeding infants perchlorate, isotopically labeled or not, in whatever amount above background levels, was not an option, we devised an approach that utilizes iodine (regardless of its chemical form), as a conservative tracer. Our null hypothesis is that perchlorate is not metabolized; it is also conserved. In solely breastfed or formula-fed infants, breastmilk or formula is the only input. If the only output occurs via urinary excretion, the ratio of concentrations of an analyte in urine to that in milk will be the same for all conserved species.

For this framework to be valid, the following will have to be true:

(a) Iodine is conserved. While there is net daily accumulation of iodine in an infant because its thyroid is growing, the daily increment of this accretion is negligible relative to daily iodine intake and excretion.

(b) For infants, urinary excretion is by far the principal mode of iodine excretion. The same must be true for perchlorate.

(c) Neither intake nor output is continuous; both are discrete events. As a constant time interval between intake and output cannot be assured, the rate of clearance of

perchlorate and iodine must be comparable to draw conclusions from a finite set of data.

Urine to Milk Concentration Ratio (UMCR) for a Conserved Species. The following are norms for infant milk intake: formula fed infants consume a greater volume; Goellner et al.[63] report average intake volumes of 657, 998, 935, and 1128 mL/d, respectively for 0-1, 1-2, 2-4 and 4-6 mo. olds, averaging 930 mL/d for 0-6 mo. olds. The same respective age groups have urinary outputs 378, 556, 496 and 505 mL/d amounting to an average urinary output of 484 mL/d for the age group. These data correspond to an intake/urine output volume ratio (the same as UMCR) of 1.7-2.2. For breastmilk, the intake volume is lower. The Child-Specific Exposure Factors Handbook[64] suggests that the mean value for a 1-3 month old is 690 mL/d, while that for a 3-6 mo. old is 770 mL/d. No data are available for urinary output volume for breastfed infants. Because there is no reason to believe that the loss of water, which takes place through breathing, sweating, feces, etc. is going to be different for breastfed vs. formula-fed infants, the UMCR for breastfed infants will be higher. Goellner's data allows us to estimate non-urine water loss for 1-3 and 3-6 mo. olds to be 440 and 531 mL/d; this will lead to respective estimates of 2.8 and 3.2 for UMCR of breastfed infants of these age groups.

The above provides approximate guidelines as to what UMCR value can be expected for a conserved analyte both for breastfed and formula-fed infants. If a particular analyte is known to be conserved, the UMCR for it can be used as a benchmark for that subject and the UMCR for another analyte can be compared to it. If the latter UMCR is significantly smaller than the benchmark UMCR, the latter species is not conserved. Regardless of the validity of these arguments, there is considerable temporal variation of perchlorate and iodine in the infant intake for a breastfed infant,

as changes in the mother's diet from one meal to the next are reflected in breastmilk composition.[18] Multiple samples per subject must therefore be analyzed.

Thus if iodine is conserved, we can take its UMCR (hereinafter called the iodine concentration factor (ICF)) as the benchmark. If UMCR for perchlorate, the perchlorate concentration factor (PCCF), is statistically indistinguishable, the null hypothesis is proven. If PCCF, however, is statistically less than ICF, the null hypothesis does not hold and perchlorate is being lost in the system.

Now we rationalize the assumptions a -c outlined above.

Iodine is Conserved. Incremental Daily Intrathyroidal Iodine Accumulation in an Infant is Negligible Relative to Daily Intake. A full-term neonate at birth has a total thyroid weight of ~1.5 g,[65,66] and based on post-mortem data, contains ~200 μg of iodine.[67] The total iodine content of a neonatal thyroid increases essentially linearly with body weight and gestational age,[67] presumably this trend continues for several months after birth. But in a euthyroid child, the thyroid does not increase in weight beyond the age of 15.[67] However, the reported iodine content of the adult thyroid for a euthyroid population varies markedly. Zabala et al.[68] report a median value of 15±8 mg in Caracas, Venezuela. Milakovic et al.[69] report a mean value of 5.2 mg in Göteborg, Sweden. Zaichick and Zaichick[67] also report an intrathyroidal iodine content of ~5 mg for 16-25 year olds in Moscow. If we assume that from birth to 6 months, the birth weight doubles (according to the Child-Specific Exposures Factors Handbook, at 6 mo., the average weight is somewhat less than twice the birth weight), and the intrathyroidal iodine content doubles, the daily accumulation rate will be <2 μg/d. A linear accumulation of intrathyroidal iodine of 15 mg over 15 years also suggests a comparable figure, 2.7 μg/d. Relative to adequate iodine intake of 110 μg/d for 0-6 mo. olds or even recommended daily allowance of 90 μg/d for 1 year olds,[70] these daily accretion rates are negligible. In fact, circulating iodine (99% of this is total thyroxine,

T4) is much larger than this; at ~560 nM as iodine,[71] assuming a 0.55 L blood volume for a 7.7 kg 7 month old, this represents 39 μg I.

Principal Iodine Excretion is Through Urine. To trace nonselective loss of iodine, the routes to loss of water also need to be considered. One major route for moisture loss is through exhaled breath. As iodine is not present in the elemental form, it is not likely that significant amounts of iodine in the vapor phase are lost through this route.

The loss of water through feces is ~5 mL/kg bodyweight/ day[72], amounting to ~37 mL for a 3-6 mo. old, compared to 505 mL urine volume per day (for a 4-6 mo. old). If the iodine content per unit water content is the same in urine and feces and urinary and fecal iodine excretion are the two major routes of iodine excretion, then 90% of excreted iodine is in the urine. The same conclusion has been drawn by others,[73,74] albeit there are relatively few direct measurements of iodine in feces.[75,76]

In athletes undertaking strenuous exercise and thereby generating a large amount of sweat, the amount of iodine loss through sweat can be significant.[74,77,78] However, this is not the case under other conditions. Estimates of transdermal loss of water (sweat, evaporation) for infants vary. Sharma et al.[72] estimate that this can be as large as twice that through feces). This is consistent with the measurements of Ariagno et al.[79] who report a normothermic evaporative loss in full-term healthy infants of 0.02 mg/cm^2/min and the estimated body surface area is 0.064 ± 0.011 m^2/kg for 0-2 year olds according to the Child-Specific Exposures Factors Handbook. Even if iodine loss in sweat is twice that in feces, urinary excretion will remain by far the dominant route of iodine excretion. This is consistent with Mao et al.'s observation[77] with soccer players in a hot humid environment that urinary iodine excretion still outweighed iodine loss through sweat by 2:1 on the average.

There may be some water loss due to regurgitative "spit-up". We were unable to find any quantitative data in the literature. It is highly unlikely that this represents

either a significant amount of liquid output compared to urinary output or a significant route of iodine and perchlorate loss.

Iodine and Perchlorate are Cleared at Comparable Rates. The clearance rate for neither perchlorate or iodine has been measured for small infants. Clewell et al.[80] have evaluated available adult data in constructing pharmacokinetic/pharmacodynamic models across life stages and suggest that the best values to use for urinary clearance are 0.1 and 0.13 L h^{-1} kg^{-1}, respectively, for iodine and perchlorate, reported for adults. It is in fact more than comparable, within the limits of significant figures, they are the same. We do not therefore expect our findings to be biased by dramatic differences in clearance rates.

Observed Iodine and Perchlorate Concentration Factors. The results for each subject, including 1 standard deviation as error bar are plotted in Figure 3. The 13 breastfed infant-mother pairs provided 43 milk samples and 39 infant urine samples, the 5 formula-fed infant-mother pairs provided 21 milk samples and 21 infant urine samples. The data were analyzed after pre-processing in 3 steps: (1) The within-subject measurements of each of the 4 variables were averaged and the standard errors of the averages were computed. (2) These averages were used to compute ICF and PCCF and also their standard deviations using the method of propagation of errors for a ratio. (3) The ICF-PCCF difference and its standard deviation for each subject were computed. These differences were analyzed by fitting the linear model:

$$d_{ij} = \mu_j + e_{ij},\ i = 1, 2, \ldots, n_j,\ j = 1, 2 \quad \ldots(1)$$

A weighted least-squares fitting was used, with the weight of d_{ij} taken as the reciprocal of the square of its standard deviation computed above. In this model, d_{ij} denotes the ICF-PCCF difference from the i^{th} subject in the j^{th} group, μ_j is the mean difference of the j^{th} group, and e_{ij} is the random error term. Here, $n_1 = 13$, $n_2 = 5$. The

model was fit using the "lm" function in the statistical software package R.[81] The fitted model was significant (p-value = 0.0004). It had a residual standard error of 1.53 with 16 degrees of freedom, and its R^2 value was 0.615. The standard model diagnostics showed that this model fit to the data was acceptable. The resulting estimates for ICF-PCCF mean differences in the two groups are summarized in the following table:

Group	Estimate	Standard error	t-value (d.f. = 16)	p-value	95% confidence interval
Breastfed	1.189	0.236	5.038	< 0.001	(0.69, 1.69)
Formula-fed	-0.054	0.127	-0.424	0.677	(-0.32, 0.22)

Table 1: Summary of results for ICF-PCCF mean difference in each group.

These results show that there is a statistically significant difference between the ICF mean and the PCCF mean (p-value < 0.001) in the breastfed group. The ICF mean is higher than the PCCF mean (95% confidence interval for mean difference = [0.69, 1.69]). On the other hand, the difference between the two means in the formula-fed group was not significant (p-value = 0.68).

These data then suggest that perchlorate is lost in the breastfed infants but may not be lost in formula-fed infants. The mean PCCF and ICF values for breastfed infants are 1.37 and 2.87, respectively. If iodine is conserved, one can estimate that on an average some 52% (= (2.87-1.37)/1.37) of the perchlorate is unaccounted for. We duly note that we are not the first to report this discrepancy. In a recently published study, based on urinary perchlorate concentrations, Valentín-Blasini et al.[82] calculated the daily average dosage for breastfed infants to be 0.42 μg/kg. They did observe that breast milk perchlorate concentrations as reported in a previous study would have

suggested a significantly higher daily dosage of 1.1 μg/kg. They chose to attribute this difference to diet, geography and small size of the studies but this difference could be just as easily interpreted if 62% of the perchlorate ingested via milk was metabolized before urinary excretion.

It is important to note that in our study formula-fed infants do not serve as controls for breastfed infants. Rather, each is an independent experiment with ICF serving as a control benchmark to determine if PCCF indicates whether perchlorate is lost. Based on volume of milk intake and urinary output we computed that the UMCR of a conserved species should be in the range of 2.8-3.2 for breastfed infants, an average ICF of 2.9±1.1 is consistent with iodine being conserved. Similarly for formula fed infants, the UMCR for a conserved species is computed to be 1.7-2.2, an ICF value of 1.2 ±0.9 is not statistically significantly different. On the other hand, the PCCF value is statistically significantly lower for breastfed infants than their ICF values, voiding the null hypothesis on perchlorate being conserved.

Note that the absolute intake of perchlorate is much lower in formula-fed babies (as has been noted also by others already[82]) and if there is any similarity to what we observe in *in-vitro* experiments, even if perchlorate is reduced at all, the extent will be much lower. In any case, whether or not any reduction of ingested perchlorate occurs with formula fed infants is of less importance as the absolute perchlorate dosage is much lower.

In retrospect, it may not be surprising that perchlorate can be metabolically reduced in the digestive systems of breastfed infants. Most processes in nature have evolved due to need. The occurrence of perchlorate in the Chilean nitrate deposits[83] has been known since the 1800's,[84] and there is strong evidence that this perchlorate was atmospherically formed.[85] Perchlorate is routinely detectable in rainwater[16,86] and traces

of perchlorate have been measured in Pleistocene and Holocene groundwater.[36] Perchlorate is not a new entity in nature; it is logical that a natural defense to its strong iodide transport inhibiting properties would have evolved to protect the newborn, the most vulnerable life stage.

Nevertheless, we hasten to add that whether this defense is adequate to handle the presence of anthropogenic perchlorate that has appeared in a population in the short term, often at much greater concentrations than the natural background, is an altogether separate question. In this limited study, we observed no correlation ($r = 0.16$) between the fraction of perchlorate lost as a function of the breastmilk perchlorate concentration. Maternal bacterial population also affects infant intestinal microflora.[55] It should not be overlooked that a large fraction of the perchlorate is excreted unchanged, nor should it be inferred that ingested perchlorate has no effects on infant iodine nutrition, especially since some absorption of perchlorate may take place before metabolization occurs. It is not our intent to suggest one should be complacent about perchlorate intake of infants.

Nevertheless, in formulating public policy, the metabolism of perchlorate by infants will need to be taken into account. Aside from reducing the steady state perchlorate concentration in the gut and thus reducing intestinal absorption, it has long been known that renal reabsorption of many anions, including iodide, occurs.[87] Logically, renal reabsorption of perchlorate must also occur. Pendrin is a membrane protein strongly expressed in the kidney;[88] it is among the known transporter of anions responsible for anion reabsorption.[89] It behaves much like an anion exchanger[90] and if so, it may provide transport/reabsorption of perchlorate even more efficiently than iodide. Intestinal metabolism may thus reduce reabsorption that will need to be considered.

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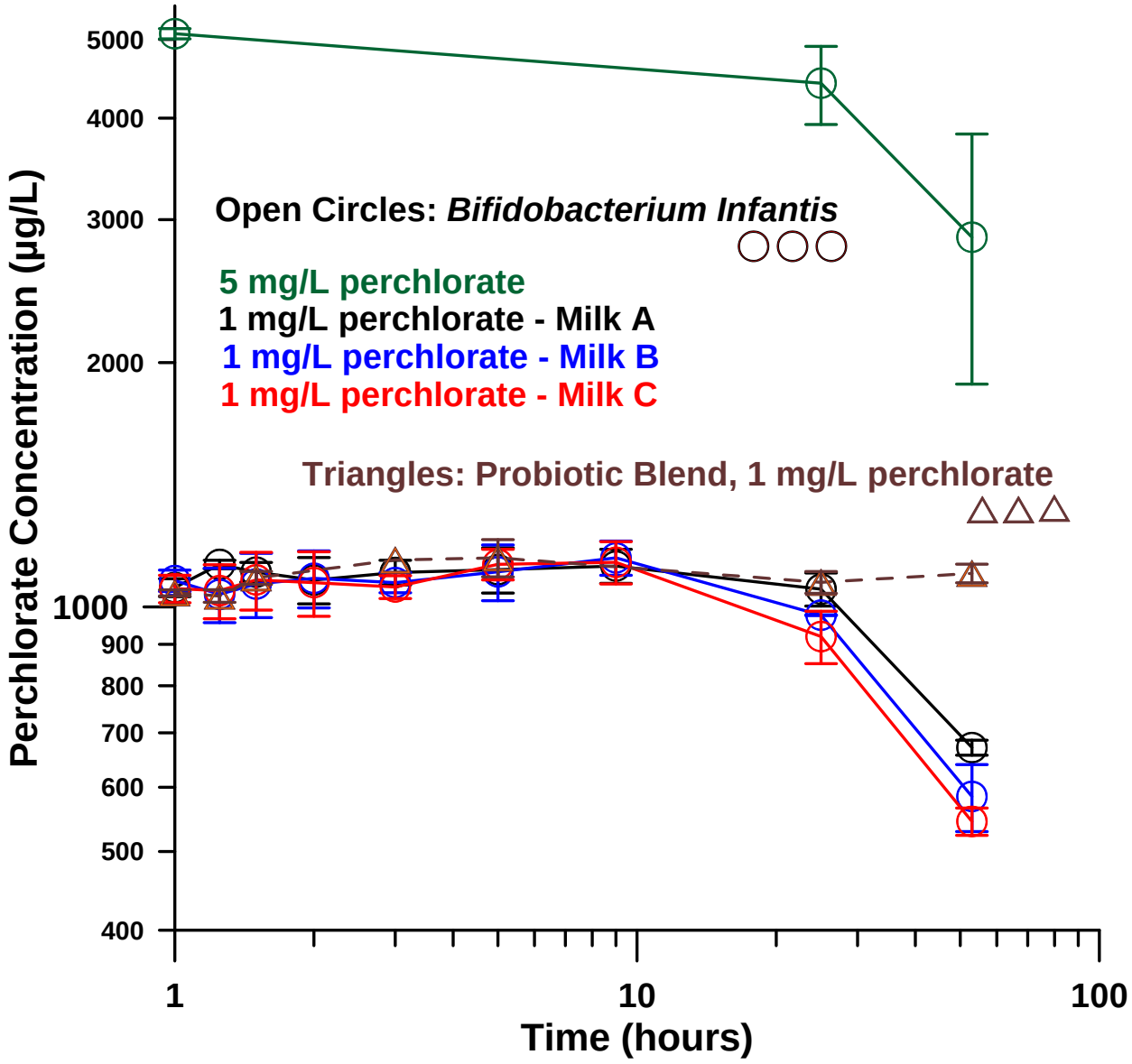

Figure 1. Perchlorate concentration in bacteria inoculated perchlorate spiked milk samples monitored up to 52 hours after bacterial addition; hour 1 represents analysis immediately after inoculation. Error bars represent one standard deviation.

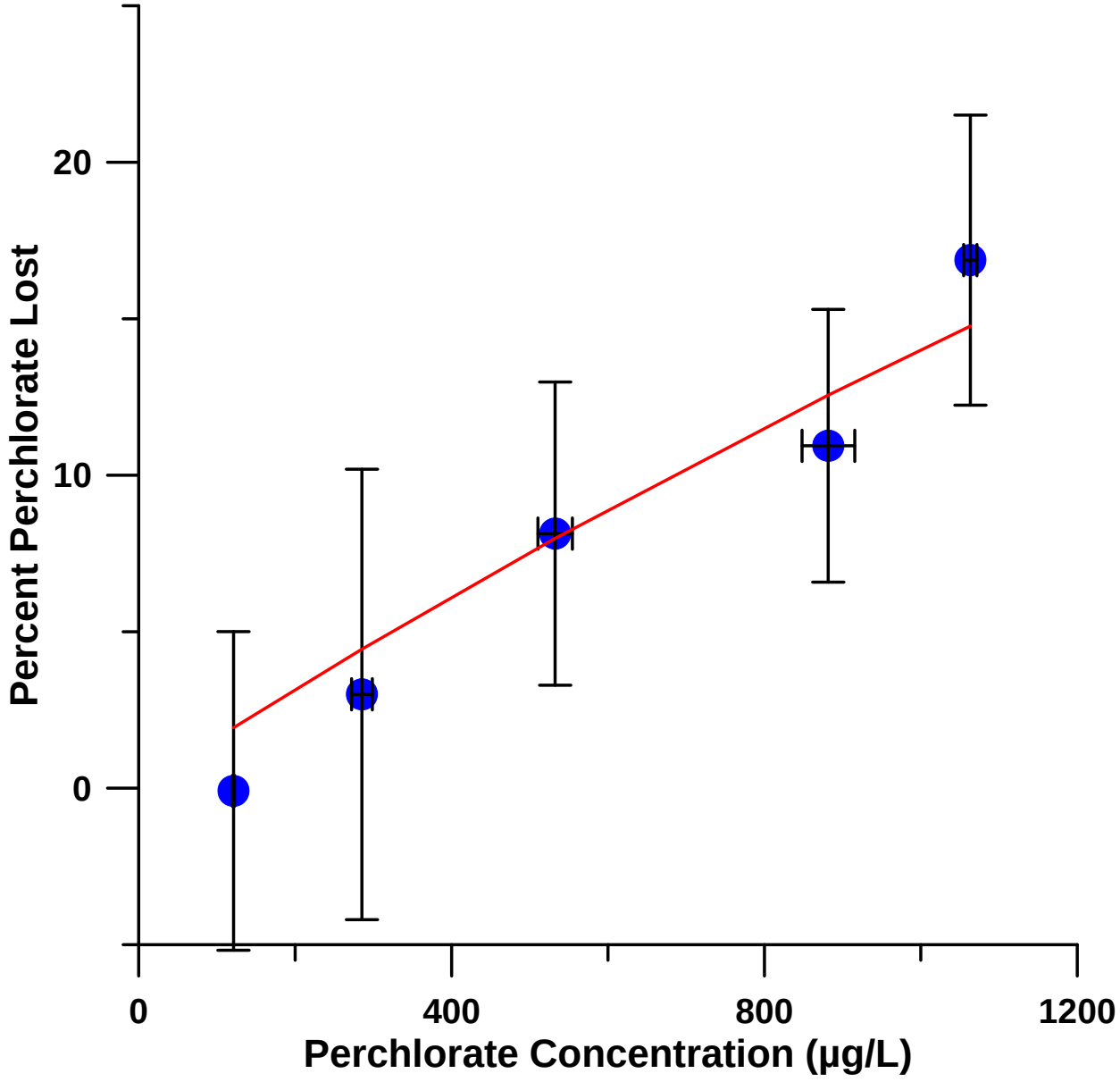

Figure 2. Percent perchlorate lost after 24 hours in milk samples spiked with various concentrations of perchlorate and incubated with *bifidobacterium infantis*. The solid line is the best fit to the model: Percent Perchlorate Loss=(a/(1+b/[ClO_4^-]).

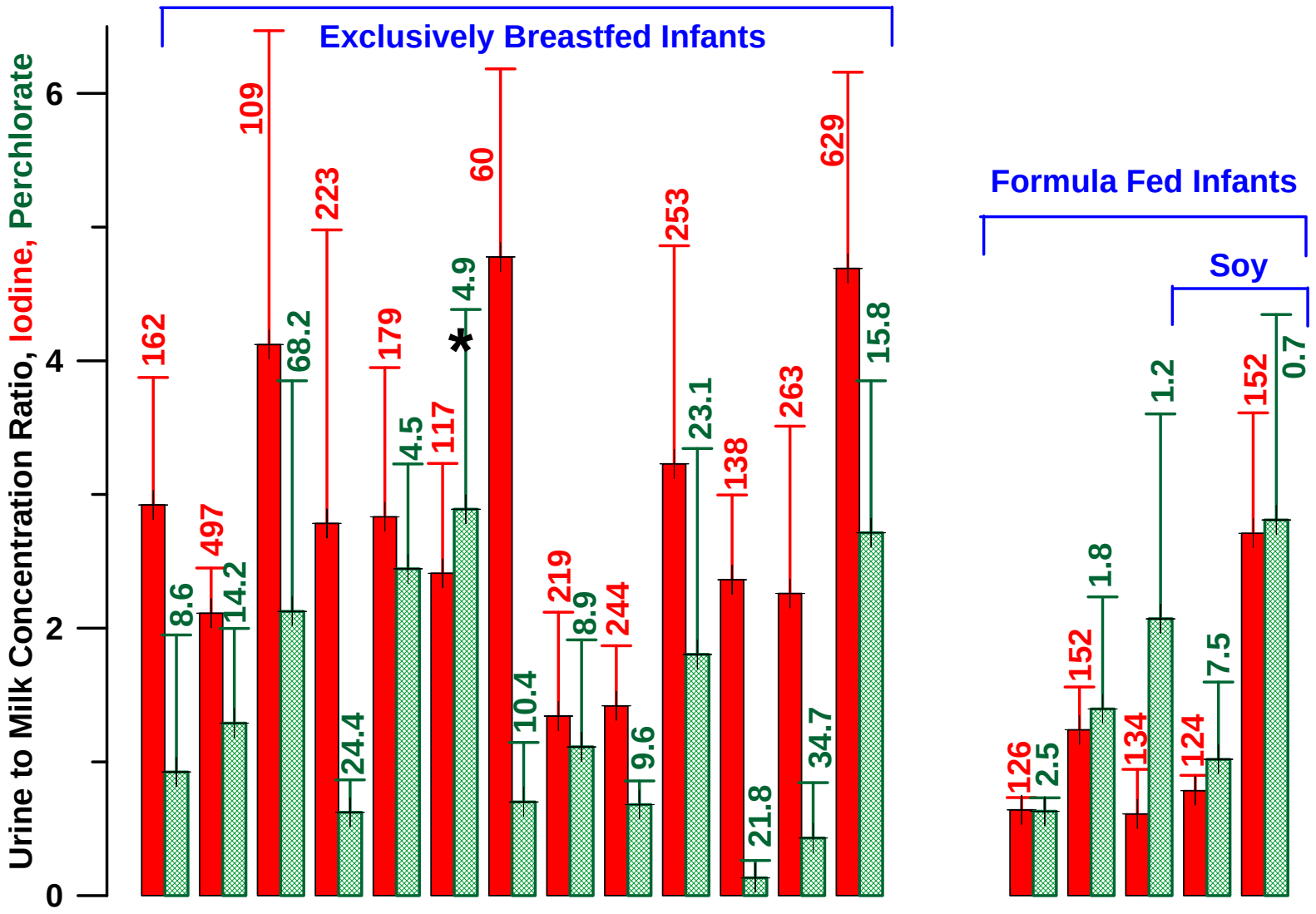

Figure 3. Iodine concentration factor, ICF, (C_{urine}/C_{Milk}) and Perchlorate concentration factor, PCCF shown as solid and crosshatched bars with 1 sd error bars for each mother-infant pair. Breastfed babies represent the group on the left, formula-fed babies on the right. For all but the asterisked subject in the breastfed group ICF exceeds PCCF while in none of the formula-fed subjects ICF exceeds PCCF. The average milk iodine and perchlorate concentrations in µg/L are noted on top of each bar. Given the ratio, the corresponding urinary values can be readily evaluated.

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