Fecal total iron Concentration is inversely associated with Fecal Lactobacillus in preschool children.

Sasank Kalipatnapu[a], Sivaraman Kuppuswamy[a], Giriprasad Venugopal[a], Venkatesh Kaliaperumal[b], Balamurugan Ramadass[b]

[a]Christian Medical College, Vellore; [b]Indian Institute of Technology, Bhubaneswar, India

Corresponding Author:

Dr. Balamurugan Ramadass
Visiting Scientist
School of Basic Sciences
Indian Institute of Technology Bhubaneswar
Odisha-752050
India
Email Address: balaramadass@iitbbs.ac.in

Acknowledgements

The Authors gratefully acknowledge Dr.SK. Dash for supporting Dr. Ramadass' Research.

The authors have no conflicts of interest to declare.

R.B. was responsible for conception of the study and over-all supervision; R.B., SK, GPV were responsible for recruitment of participants and sample collection; Sivaraman K, GPV, V.K. was responsible for sample processing and analysis; R.B. was responsible for funding; and S. K, V.K. and R.B. were responsible for writing the manuscript. We thank Priya for dietary survey.

Abstract

Background: Iron deficiency is associated with stunting and poor performance in children. Oral iron supplementation is widely promoted to correct iron deficiency. However, excess iron may be toxic to beneficial luminal gut bacteria and could support growth of pathobionts.

Objective: To analyze the fecal total iron concentration and fecal Lactobacillus levels in a cohort of stunted and normal children.

Design: The study was undertaken in two different locations. One of them is a rural area and other is a semi-urban-slum area, both areas are located in the Vellore district of Tamilnadu state. 20 children (10 stunted and 10 normal growth) aged 2 to 5 years from each area were recruited. Both groups were nearly identical demographically. Fecal samples were collected. Fecal total iron was estimated, fecal DNA was extracted and subjected to 16S rDNA-targeted real-time polymerase chain reaction to determine the relative predominance of *Lactobacillus* and *Escherichia coli*.

Results: The fecal total iron concentration in rural children (3656 µg/ g wet wt. of feces) was significantly higher when compared to semi-urban-slum children (114.9 µg/ g wet wt. of feces, $p<0.005$). Inversely, fecal Lactobacillus in rural children (Median 3.18×10^{-3} Relative difference compared with total bacteria) was significantly lower when compared to semi-urban-slum children (Median 59.33×10^{-3}, $p<0.005$). There was no significant change observed between normal and stunted children. E.coli levels remained unaffected.

Conclusion: The present study documents an inverse relationship between fecal iron concentration and Fecal *Lactobacillus* concentration in children belonging to two different localities independent of their nutritional status.

Keywords: Nutrient transport and metabolism, Probiotics/intestinal host defense, Microbial pathogenesis

What is known?

- Iron is an essential micronutrient necessary for a variety of biological functions.
- <10% of ingested iron gets absorbed, while the remaining iron overflows to the colon.
- Excess iron may be toxic to beneficial luminal gut bacteria.
- Fecal *Lactobacillus* was found to be reduced in anemic young women when compared to women without anemia.

What is new?

- First Human study to relate Fecal Iron estimation to the Fecal *Lactobacillus*.
- Fecal iron has an inverse relationship with fecal *Lactobacillus* levels in children.
- Utility of fecal total iron estimation should be further evaluated as a useful tool in clinical nutrition:
    A. To rationalize Iron fortified food supplements.
    B. To screen in developing countries where empirical iron supplementation is part of National Health Program.

INTRODUCTION

Iron is an essential micronutrient for almost every form of life on the planet. Availability of iron is necessary for a variety of biological functions, especially physical growth and hematopoiesis[1]. Iron deficiency in children is found to correlate with decreased cognitive performance and may include weakness, fatigue, and inability to focus. Iron deficiency, when severe, is correlated with increased risk of preterm labor, low birth weight, child and maternal mortality[2]. Oral iron supplementation is recommended as simple and effective way to correct iron deficiency. However, excess iron may be extremely toxic to aerobic organisms due to the formation of reactive oxygen species which can damage nucleic acids and other cellular components. For this reason, iron uptake and cellular iron reserves are tightly regulated in aerobic organisms[3][4]. The human body tightly regulates iron levels at the level of intestinal absorption, as there is no active mechanism for iron efflux. Iron absorption

occurs in the small intestine, where dietary iron is enzymatically reduced from ferric iron to ferrous iron, and then transported inside the cells[5][6]. Excess iron is tightly bound to transferrin and lactoferrin for safe transportation across the body. Interestingly, human body sequestrates iron to defend itself from most invading pathogens, as they need iron for rapid growth during infection[7][8][9].

<10% of ingested iron gets absorbed, while the remaining iron overflows to the colon and is available to the residing microbiota [6]. The human body plays host to a diverse community of microbes. These resident microbes, approximately 10^{14} in number, with their nucleic material are collectively described as the human microbiome. Dietary habits greatly influence the composition of the gut microbiome. Maintenance of a healthy human gut microbiome is essential for health[10][11][12]. In our previous study, we showed that the fecal population of *Lactobacillus* was reduced in anaemic young women[13]. However, the interaction between fecal total iron concentration and Fecal *Lactobacillus* population was not elucidated.

The major source of luminal iron is likely to be excess 'leftover' iron that is not absorbed from the diet. This iron acts as a growth factor for bacteria that are dependent on iron for their growth. Iron is an absolute growth requirement for several species of pathogenic bacteria[14][15], and it is possible that oral iron supplementation may increase the abundance of pathogenic bacteria in the feces. In the present study conducted in preschool children, we hypothesized that the concentration of fecal total iron has a predictable association with Fecal *Lactobacillus* population.

MATERIALS AND METHODS

Study area: The study was undertaken in two different areas located 30 kms apart from each other. One of them is a rural area (Kilvalam) and other is a semi-urban-slum area (Samuel Nagar), both areas located within Vellore district of Tamil Nadu state. The present study was approved by the institutional review board of the Christian Medical College, Vellore. Informed consent was obtained from the parents of the participants prior to recruitment.

Participants: Twenty children, aged 2 to 5 years from each area were recruited. Baseline data about the demographic details and anthropometric measures was collected during study recruitment. Ten of the children from each area were recruited with normal (mean $\pm$ one standard deviation) height for age as per the WHO growth charts, while the other 10 were recruited stunted with height for age less than 2 standard deviations below the mean. Children with any chronic illness, peripheral edema, antibiotic treatment in the last three months, diarrheal episodes in the preceding week or on iron supplementation in the last one month or a height for age score greater than 3 SD from the reference value, as well as children between 1 and 2 SD from the mean on either side, were excluded from the study.

Usual diet intake and specifically iron intake was assessed in all the study participants using 3-day dietary recall and then analyzed for content using reference nutrient values for Indian foods.

Sample collection: Fecal samples were collected from 40 children from both the localities and were immediately transported to the lab on ice and stored at -80°C until analysis.

Fecal Total Iron estimation: Freeze-dried fecal samples from all 40 children were analysed. 100-200 mg of freeze-dried Feces was placed in a crucible and was burnt to ash in a muffle furnace at 450˚C for 12 hours.

Dry ash was transferred into a clean 1.5 ml Axygen microcentrifuge tube (Corning, USA) and weighed using microbalance. 1 ml of 2N HCl was added to the ash and mixed vigorously using the vortex to dissolve. Iron standard (10µg/mL) was prepared from the laboratory stock $fecl_3$ reagent. Dissolved ash was used to estimate fecal total iron using Ferene (Chromogen) end-point method (Fisher diagnostics, VA, USA). Chromogen is allowed to react with reduced iron (in reduced pH) to produce Iron-Chromogen complex. This complex has a visible range spectrum with peaks in the 500-600nm range, which is measured spectrophotometrically and is proportional to the total iron content. Measured Fecal total iron levels were expressed as µg of iron/ gram wet wt. of feces [16].

Quantitation of Fecal bacterial population: Fecal DNA was extracted using two successive rounds of bead beating with 0.1 mm zirconium–silica beads (Biospec Products, Bartlesville, OK, USA) followed by extraction with a QIAamp DNA Stool Minikit (Qiagen, Germantown, MD). The extracted DNA was then subjected to quantitative PCR amplification using genus-specific primers targeted at 16S ribosomal DNA of *Lactobacillus* and *E.coli*. The total bacterial load of the sample was also amplified using conserved universal primers. Primers, PCR conditions & results were expressed as described previously(12).

Statistics: The demographics were analyzed using descriptive statistics and the values were expressed as Mean ± Standard Deviation. Fecal total iron concentration, Fecal *Lactobacillus* population, and Fecal *E.coli* population were expressed as median and interquartile ranges (IQR). The significance of differences between groups was assessed using Mann Whitney U-test (with logarithmic transformation in case of both the bacteria and Fecal Iron). P value <0·05 was considered statistically significant.

RESULTS

The parents of the forty children (20 with normal and 20 stunted HAZ score) from two different area consented for participation in this study. There were a total of 18 boys and 22 girls with an equal number of children in the healthy and stunted groups. A total of 40 fecal samples were collected and analyzed. Table 1 shows the characteristics of all the 40 children. There were significant differences between healthy and stunted children in their HAZ scores.

Fecal total iron & microbes*:* Fecal total iron concentration did not differ significantly between stunted (736.3, IQR 94.4-3240) and normal children (1520, IQR 183.6-4182). It was significantly lower in Semi-urban-slum children ($p<0.005$) when compared to rural children (Figure 1). Dietary iron consumption in both the groups were similar (Table 2). There were no significant changes between Genders. However, dietary iron consumption in both the groups were below the Recommended Dietary Allowance.

Fecal *Lactobacillus* did not differ significantly between stunted (Median 7.6×10^{-4}, IQR 1.9×10^{-4} - 8.2×10^{-3}) and normal children (Median 3.8×10^{-3}, IQR 7.9×10^{-4}- 1.4×10^{-2}). It was significantly higher in Semi-urban-slum children ($p<0.005$) when compared to rural children (Figure 2). However, Fecal *E.coli* abundance did not vary significantly.

The correlation between Fecal total Iron and lactobacillus abundance across all children from both rural and semi-urban-slum area is depicted (Figure 3). In the children from the two areas, there was a trend for inverse correlation between the fecal total iron and Lactobacillus abundance.

Discussion

This study demonstrates a significant increase in fecal total iron and a reduction in Fecal Lactobacillus abundance in rural pre-school children and vice versa in semi-urban-slum pre-school children of southern India. By contrast, there was no change in Fecal E.coli abundance in both the study groups.

In a preceding observational study, we have shown decreased Fecal *Lactobacillus* abundance in anemic women of reproductive age. The reason for the reduction in *Lactobacillus* abundance remained unanswered. In this study, we hypothesised that fecal iron will alter based on the growth status of the children, and this in turn will determine the Fecal *Lactobacilli* concentration. To address this we recruited children from two different localities with differences in growth status. The children from both localities were homogenous with respect to their dietary iron intake and socio-economic status. Interestingly, fecal total iron concentrations significantly varied in children based on their localities and remained unchanged with differences in growth status or gender. It is, therefore, reasonable to speculate on the intake of dietary fiber (soluble & insoluble), non-digestible disaccharide and oligosaccharides. Based on dietary questionnaire, all the forty children had no significant difference in thier dietary pattern. Consumption of meat, milk & milk products, energy were all uniform. However specific soluble or insoluble fibre content of the diet was not quantified in this study.

Approximately 40% of the pre-school children in India are moderately anemic and about equal number of them are moderately stunted. Iron deficiency anemia reduces the learning capacity, causing low intelligence, and ultimately impacting the growth of the country[17][18]. All children recruited in this study were apparently healthy, asymptomatic and school going. The human gut microbiome is highly dynamic during early childhood. Maturing gut microbiome highly relies on a variety of inter and intra generational factors like, maternal nutrition before, during & immediately after pregnancy, mode of delivery, duration of breast feeding, weaning and other domestic factors. However, diet and dietary supplementation play a predominant role in shaping the maturing gut microbiome [19][20][21]. This study highlights the influence of iron over Fecal *Lactobacillus* abundance due to factors that are specific to the rural set-up. Measuring fecal occult blood, parasites and calprotectin could have ruled out few factors that are specific to rural population, and this clearly is a limitation.

Increased fecal total iron had no effect on *E.coli* abundance, while it did not favor the growth of *Lactobacillus* population. This effect may be due to their differential iron requirement for growth, as they rely on fermentative metabolism for their growth. An *in vitro* study established an association between low luminal iron levels and *Lactobacilli* abundance[22]. Our study is the first human study to document an inverse relationship between fecal *Lactobacilli* and fecal iron. *Lactobacillus* in the colon has been shown to promote beneficial (probiotic) effects locally. They have anti-inflammatory properties and form a barrier against pathogen colonisation. Decreased *Lactobacillus* abundance in these children may negatively influence their maturing gut microbiome[23][24][25]. Considering the effects of dietary iron over-flow on the *Lactobacillus* abundance, it is possible that empirical iron supplementation to children across the world might have a greater impact on childs gut microbiome, growth and development.

To our knowledge, this is the first study to estimate fecal total iron and to document its association with fecal *Lactobacillus* abundance. Emperical iron supplementation is a worldwide strategy to treat iron deficiency. However in light of the aforementioned association, utility of fecal iron estimation at various settings should be evaluated. It may be a useful tool to plan effective iron supplementation or iron fortification strategies. Given the strength and nature of this inverse relationship, it may be prudent to consider the use of a probiotic supplementation to replenish *Lactobacillus* in conjucntion with iron supplementation.

Conclusion

The present study documents an inverse relationship between fecal total iron concentration and Fecal *Lactobacillus* abundance in children from two different localities independent of their growth status. In a relatively homogenous population, the fecal total iron concentration was significantly higher in children from rural area. Concomitantly, the proportion of *Lactobacillus* in Feces was found to be lower in rural areas when compared to Semi-urban-slum area.

Reference

1. Hunt JR, Roughead ZK (1999) Nonheme-iron absorption, fecal ferritin excretion, and blood indexes of iron status in women consuming controlled lactoovovegetarian diets for 8 wk. Am J Clin Nutr 69:944–952.

2. Kassebaum NJ, Jasrasaria R, Johns N, et al (17AD) A systematic analysis of global anaemia burden between 1990 and 2010. Lancet 381, Suppl:S72. doi: http://dx.doi.org/10.1016/S0140-6736(13)61326-4

3. Kortman GAM, Raffatellu M, Swinkels DW, Tjalsma H (2014) Nutritional iron turned inside out: Intestinal stress from a gut microbial perspective. FEMS Microbiol Rev 38:1202–1234. doi: 10.1111/1574-6976.12086

4. Imlay JA (2006) Iron-sulphur clusters and the problem with oxygen. Mol Microbiol 59:1073–1082. doi: 10.1111/j.1365-2958.2006.05028.x

5. Kühn LC (2015) Iron regulatory proteins and their role in controlling iron metabolism. Metallomics 7:232–243. doi: 10.1039/C4MT00164H

6. Sharp P, Srai S-K (2007) Molecular mechanisms involved in intestinal iron absorption. World J Gastroenterol 13:4716–4724.

7. Nakashige TG, Zhang B, Krebs C, Nolan EM (2015) Human calprotectin is an iron-sequestering host-defense protein. Nat Chem Biol 11:765–771. doi: 10.1038/nchembio.1891

8. Bullen JJ, Rogers HJ, Spalding PB, Ward CG (2006) Natural resistance, iron and infection: A challenge for clinical medicine. J Med Microbiol 55:251–258. doi: 10.1099/jmm.0.46386-0

9. Weinberg ED (2009) Iron availability and infection. Biochim Biophys Acta - Gen Subj 1790:600–605. doi: 10.1016/j.bbagen.2008.07.002

10. Wu GD, Chen J, Hoffmann C, et al (2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105–8. doi: 10.1126/science.1208344

11. Arumugam M, Raes J, Pelletier E, et al (2011) Enterotypes of the human gut microbiome. Nat May 12:174–180. doi: 10.1038/nature09944

12. Ramakrishna BS (2013) Role of the gut microbiota in human nutrition and metabolism. J Gastroenterol Hepatol 28:9–17. doi: 10.1111/jgh.12294

13. Balamurugan R, Mary RR, Chittaranjan S, et al (2010) 106. Low levels of faecal lactobacilli in women with iron-deficiency anaemia in south India. Br J Nutr 104:931–4. doi: 10.1017/S0007114510001637

14. Kortman GAM, Boleij A, Swinkels DW, Tjalsma H (2012) Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PLoS One. doi: 10.1371/journal.pone.0029968

15. Zimmermann MB, Chassard C, Rohner F, et al (2010) The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. Am J Clin Nutr 92:1406–1415. doi: 10.3945/ajcn.110.004564

16. Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT (1999) Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am J Clin Nutr 69:250–255.

17. Singh RK, Patra S (2014) Extent of Anaemia among Preschool Children in EAG States, India: A Challenge to Policy Makers. Anemia 2014:868752. doi:

10.1155/2014/868752

18. Amrutha Veena K, Kowsalya S, Kothandapani S (2014) Micronutrient malnutrition profile of infants in South India. J Hum Nutr Diet 27:54–57. doi: 10.1111/jhn.12101

19. Ahmed T, Auble D, Berkley JA, et al (2014) An evolving perspective about the origins of childhood undernutrition and nutritional interventions that includes the gut microbiome. Ann N Y Acad Sci 1332:22–38. doi: 10.1111/nyas.12487

20. Balamurugan R, Janardhan HP, George S, et al (2008) Bacterial succession in the colon during childhood and adolescence: Molecular studies in a southern Indian village. Am J Clin Nutr. doi: 10.3945/ajcn.2008.26511

21. Dinh DM, Ramadass B, Kattula D, et al (2016) Longitudinal analysis of the intestinal microbiota in persistently stunted young children in south India. PLoS One. doi: 10.1371/journal.pone.0155405

22. Dostal A, Fehlbaum S, Chassard C, et al (2013) Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. FEMS Microbiol Ecol 83:161–175. doi: 10.1111/j.1574-6941.2012.01461.x

23. Mortaz E, Adcock IM, Ricciardolo FLM, et al (2015) Anti-inflammatory effects of Lactobacillus Rahmnosus and Bifidobacterium breve on cigarette smoke activated human Macrophages. PLoS One. doi: 10.1371/journal.pone.0136455

24. Cox MJ, Huang YJ, Fujimura KE, et al (2010) Lactobacillus casei abundance is associated with profound shifts in the infant gut microbiome. PLoS One. doi: 10.1371/journal.pone.0008745

25. Omar JM, Chan YM, Jones ML, et al (2013) Lactobacillus fermentum and Lactobacillus amylovorus as probiotics alter body adiposity and gut microflora in healthy persons. J Funct Foods 5:116–123. doi: 10.1016/j.jff.2012.09.001

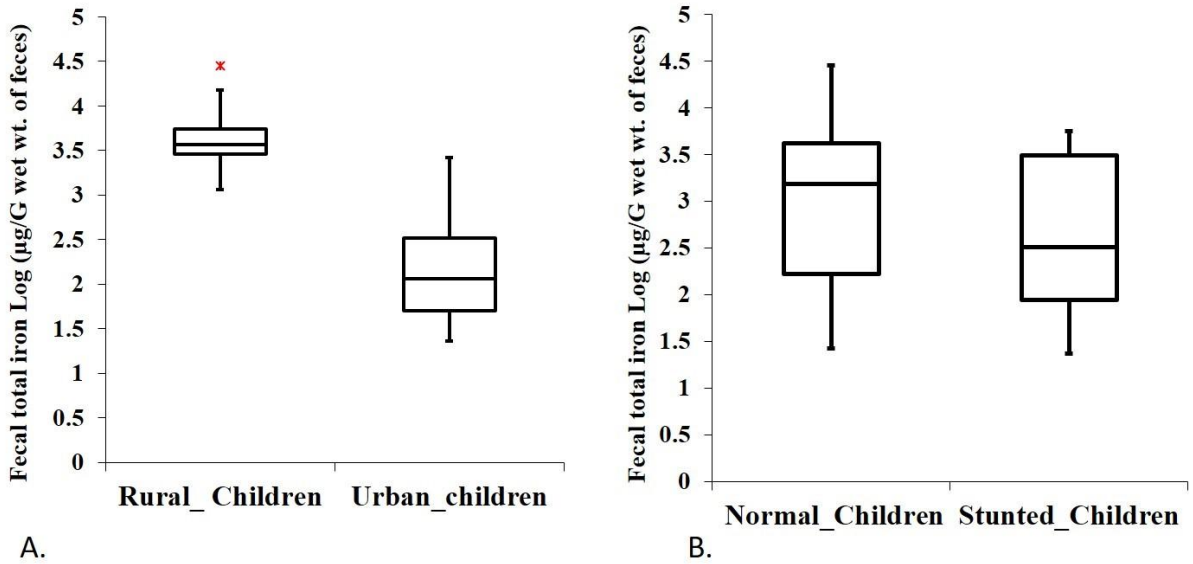

Figure 1: Fecal Total Iron (µg/ G wet wt. of feces) Median and inter quartile ranges (IQR) in log scale is plotted. A. Significant difference in Fecal Total iron observed between Urban & Rural Children (P<0.005, Mann Whitney test). While B. No significant change between Normal Children Vs. Stunted Children

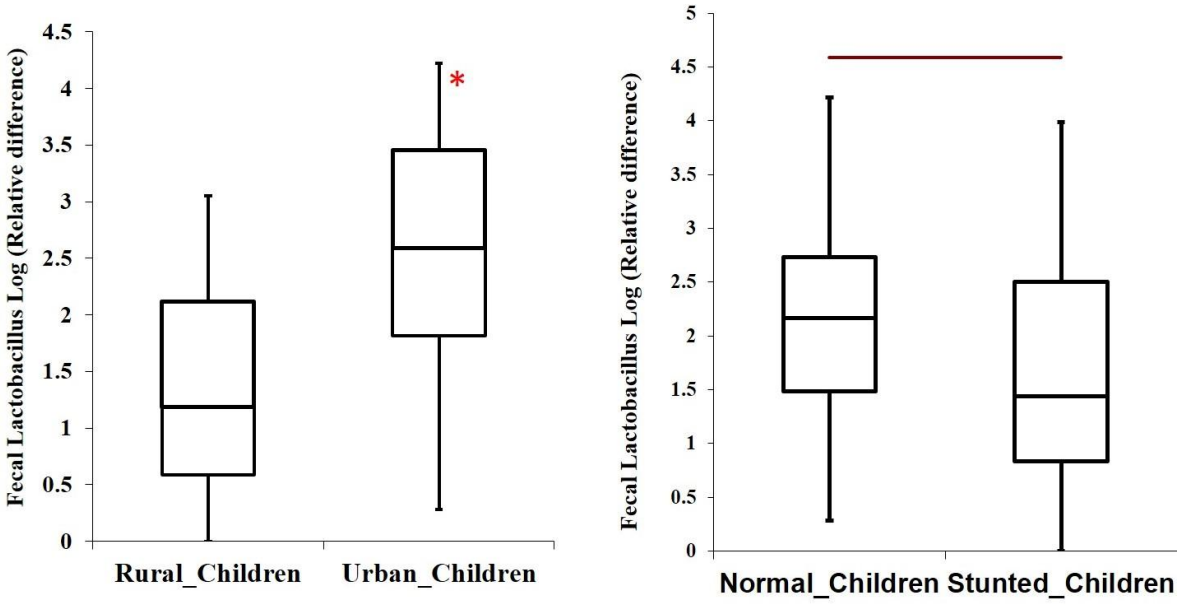

Figure 2: Fecal Lactobacillus (Relative difference), Median and inter quartile ranges (IQR) in Log scale is plotted. A. Significant difference in Fecal Total iron observed between Urban & Rural Children (P<0.005, Mann Whitney test). While B. No significant change between Normal Children Vs. Stunted Children

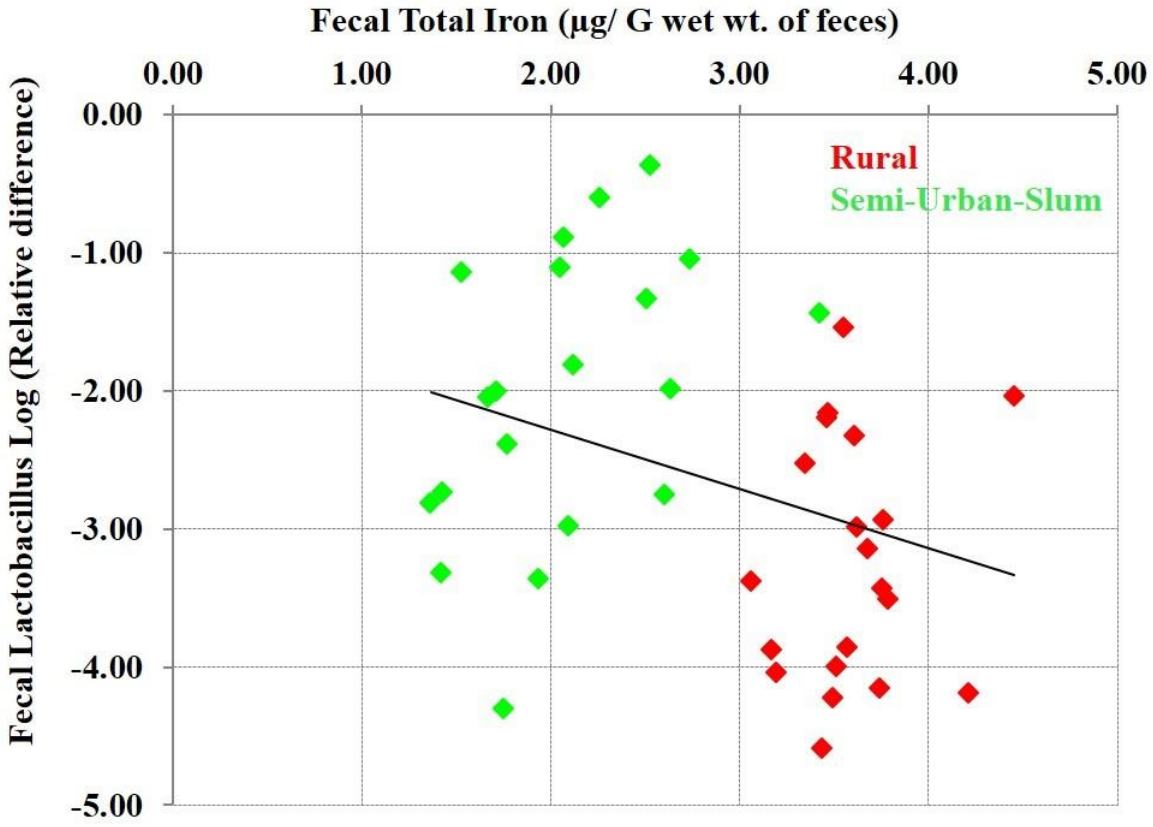

Figure 3: Correlation between the Fecal total Iron and lactobacillus abundance across all children from both rural and semi-urban-slum area is plotted. The abscissa and ordinate show, respectively, the Fecal total Iron and lactobacillus abundance relative to the total number of bacteria. The color code: Semi-Urban-Slum (Green) to Rural (red) Children.

Table 1: Characteristics of study Children from both Rural and Urban-slum, the two cohorts were similar in all aspects.

Characteristic	**Rural**		**Urban**	
No of children	20		20	
No of stunted children (HAZ <-2)	10		10	
No of normal children	10		10	
Average Height	94.03 ± 8.2 cm		94.5 ± 7.03 cm	
Average Weight	12.9 ± 2.5 kg		13.1 ± 2.1 kg	
Children	Male	Female	Male	Female
	9	11	9	11
Average height	97.6 ± 8.02	91.06 ± 7.6	96.7 ± 7.02	92.7 ± 6.8
Average weight	14.4 ± 2.6	11.6 ± 1.7	13.9 ± 1.3	12.5 ± 2.
Children	Normal	Stunted	Normal	Stunted
Average height	99.9 ± 6.04	88.2 ± 5.7	99.3 ± 6.5	89.2 ± 3.4
Average weight	14.6 ± 1.9	10.9 ± 1.4	14.5 ± 1.7	11.7 ± 1.3

Table 2: Fecal iron and lactobacillus proportions in children (Based on Locality).

	Rural	**Urban**	**P-value**[1]
Age in month, mean (SD)	40.0 (7.6)	41.9 (13.3)	0.93
Male (%)	45	45	-
Iron intake from diet/day (mgs), Median (IQR)	2.3 (1.8-3.1)	2.6 (1.8-3.6)	0.29
Fecal parameters			
Fecal Iron Concentration (µg of iron/ gram wet wt. of feces), Median (IQR)	3656 (2860-5557)	114.9 (50-327)	<0.005
Fecal Lactobacillus (Relative difference)**,** Median (IQR)	3.9×10^{-4} ($9.8 \times 10^{-5} – 7.4 \times 10^{-2}$)	1.01×10^{-2} ($1.7 \times 10^{-3} – 7.4 \times 10^{-2}$)	<0.05
Fecal E.coli (Relative difference)**, Median**	4.059×10^{-5}	6.548×10^{-5}	0.23