Short Communication

Low levels of faecal lactobacilli in women with iron-deficiency anaemia in south India

Ramadass Balamurugan, R. Regina Mary, Sucharita Chittaranjan, Hepsiba Jancy, R. Shobana Devi and Balakrishnan S. Ramakrishna*

Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Vellore 632 004, India
(Received 7 January 2010 – Revised 30 March 2010 – Accepted 31 March 2010 – First published online 7 May 2010)

Fe deficiency in women contributes significantly to maternal and child morbidity in India. The intestinal bacterial flora may facilitate absorption of Fe from the caecum and proximal colon. The present study investigated the possibility that intestinal microbiota of anaemic young women may differ from that of women with normal Hb levels. The microbiota was quantified by real-time PCR in faeces of eight anaemic (Hb ≤ 100 g/l) and twenty-six normohaemic (Hb ≥ 120 g/l) women aged 18–25 years. Sequences of 16S ribosomal DNA (rDNA) specific to Bifidobacterium genus, Lactobacillus acidophilus group, Bacteroides–Prevotella–Porphyromonas group, Clostridium leptum group and Eubacterium rectale were amplified and expressed (as relative difference) relative to the universally conserved bacterial 16S rDNA sequences. Dietary intakes of energy, carbohydrate, fibre and Fe were similar in both the groups. Fe deficiency in young women in south India was associated with low levels of lactobacilli in the faeces. The relationship between lactobacilli and Fe deficiency needs to be explored further.


Fe, an essential trace element, is required for a wide spectrum of biological functions. Women in the reproductive age group in low-income countries are particularly at risk for Fe deficiency and anaemia. Up to 85% of women in the reproductive age group in rural India have Hb levels <120 g/l(1), potentially resulting in adverse pregnancy outcomes, reduced productivity at work and impaired physical capabilities(2,3). Biofortification of commonly eaten foods with Fe is a practical approach to intervention in this target group(2,4,5). In a cohort of non-pregnant, non-lactating women aged 18–35 years in south India, mean dietary Fe intake was 9.5 mg/d predominantly from consumption of cereals, pulses and vegetables, but only 2.8% of non-haem Fe was bioavailable(5) because of the presence of phytates that inhibit duodenal Fe absorption. Fe is absorbed primarily from the duodenum. Recent studies suggest that Fe is also absorbed from the proximal colon, with such absorption being enhanced by the presence of easily fermentable carbohydrates such as inulin(6,7). Inulin and other prebiotic carbohydrates stimulate the growth of bacteria that produce SCFA such as propionic acid, which in turn enhances mineral uptake(8). Rats chronically fed carbohydrates, which results in propionic acid fermentation, show an accumulation of Fe in their caeca(9).

We hypothesised that the faecal flora of young women with Fe deficiency may be deficient in certain groups of bacteria that produce SCFA, and we undertook to investigate this by conducting a case–control study in south India.

Methods

The participants were young women aged between 18 and 25 years residing in a student hostel in Vellore. After focus group discussions with the students and their teachers, eligible individuals were invited to participate. Hb was estimated using an automated counter, and interested individuals with Hb levels ≤100 or ≥120 g/l were enrolled into the study. Participants were excluded if they had consumed antibiotics within the last 1 month, or if they had a history of menstrual disturbance. All participants maintained a detailed diet diary for 1 week after initial training in its use. The diaries were reviewed daily by an investigator (J. H.) in order to ensure that the dietary intake was adequately recorded. Food quantities were measured according to a standard set of cups and spoons, and the daily intake of the relevant nutrients was calculated from standard tables for the composition of Indian foods(10). The socioeconomic status of the participant’s family was

* Corresponding author: B. S. Ramakrishna, email rama@cmcvellore.ac.in
assessed using a questionnaire standardised for the Indian rural population\textsuperscript{(11)}.

Samples of venous blood were taken for the estimation of Hb and Fe. Each participant provided a sample of freshly passed stool in the morning, and this was transported to the laboratory on ice and stored in aliquots at $-80^\circ$C until DNA extraction. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the institutional review board of the Christian Medical College. Informed written consent was obtained from the participants.

Faecal DNA was extracted using QiaAmp stool DNA minikits (Qiagen, Hilden, Germany). Faecal bacteria were analysed by quantitative PCR using primers targeted at 16S ribosomal DNA of \textit{Escherichia coli}, \textit{Clostridium leptum} group, \textit{Bifidobacterium} genus, \textit{Bacteroides--Prevotella--Porphyromonas} group and \textit{Lactobacillus acidophilus} group. The specificities of these primers and the PCR conditions have been reported earlier\textsuperscript{(12)}. The \textit{L. acidophilus} group primers detected \textit{Lactobacillus acidophilus}, \textit{Lactobacillus amylovorus}, \textit{Lactobacillus amylocticus}, \textit{Lactobacillus crispatus}, \textit{Lactobacillus gasser} and \textit{Lactobacillus johnsonii}. The bacterial groups chosen for study represent numerically important constituent classes of faecal bacteria found in this age group. Lactobacilli, though much less numerous, are widely considered to be beneficial to human health in many ways. All of them significantly impact bacterial fermentation of carbohydrates in the colon. Real-time PCR was done in a Chromo4 system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green master mix (Eurogentec, Liege, Belgium) as we have described earlier\textsuperscript{(12)}. Conserved 16S ribosomal DNA sequences found in all bacteria (‘universal’ sequences conserved for domain bacteria) were amplified simultaneously using appropriate primers, and they served as the denominator, against which quantitative amplification of the other 16S ribosomal DNA amplicons was reported. DNA copy was expressed not as an absolute copy number but as a ‘relative difference’, i.e. the cycle threshold at which DNA for each target was detected relative to the cycle threshold at which ‘universal bacterial’ DNA was detected upon amplification\textsuperscript{(12)}. The relative difference was automatically calculated using the Opticon 3.1 software available with the Chromo4 when the universal amplicon was set as the reference. The reference amplicon was assigned a value of 1, and target bacterial groups were expressed as decimal values in proportion to this.

Statistics

Values were expressed as median (range or interquartile range). Significance of differences between groups was assessed using unpaired $t$ test (with logarithmic transformation in case of bacteria). A two-tailed $P$ value $<0.05$ was considered statistically significant.

Results

Of the 120 individuals screened after the focus group discussion, nine had Hb levels $<100$ g/l, twenty-two had Hb

\begin{table}
\centering
\caption{Demographic characteristics of study participants (Median values with their ranges)}
\begin{tabular}{llll}
\hline
 & Anaemic (n 8) & Normohaemic (n 26) & \\ 
 & Median & Range & Median & Range & $P$ \\
\hline
Height (cm) & 150 & 143–160 & 156 & 150–161 & NS \\
Weight (kg) & 46 & 42–50 & 45 & 36–54 & NS \\
BMI (kg/m$^2$) & 20-5 & 17.9–22.2 & 19.0 & 14.6–24.8 & NS \\
Socioeconomic class* & II & II–II & II & I–III & NS \\
Hb (g/l) & 90 & 76–96 & 136 & 130–151 & $<0.0001$ \\
Serum Fe ($\mu$g/l) & 170 & 120–350 & 550 & 220–1190 & $<0.0001$ \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Dietary intakes of study participants (Median values and ranges)}
\begin{tabular}{llll}
\hline
 & Anaemic (n 8) & Normohaemic (n 26) & \\ 
 & Median & Range & Median & Range & $P$ \\
\hline
Fe (mg/d)* & 8.8 & 6.7–12.9 & 8.9 & 5.2–14.6 & NS \\
Energy (kJ/d) & 6016 & 5071–10175 & 6091 & 3807–8238 & NS \\
Total dietary fibre (g/d) & 2.9 & 2.1–3.9 & 3.6 & 2.0–5.7 & NS \\
Insoluble fibre (g/d) & 0.5 & 0.1–1.6 & 0.8 & 0.2–3.3 & NS \\
Carbohydrate (g/d) & 256 & 198–273 & 220 & 149–476 & NS \\
Complex carbohydrate (g/d) & 220 & 155–273 & 220 & 149–476 & NS \\
Milk and milk products (g/d)† & 75 & 0–100 & 30 & 0–95 & NS \\
\hline
\end{tabular}
\end{table}

\textsuperscript{*} Median intake of haem Fe was nil in both the groups. Only one participant in the anaemic group and three participants in the reference group ate any meat or fish.

\textsuperscript{†} Only one individual in the anaemic group and two individuals in the reference group consumed fermented milk products.
levels of 100–120 g/l and the rest had Hb levels > 120 g/l. Thirty-four participants were included in the study, eight participants with Hb < 100 g/l and twenty-six participants with Hb > 120 g/l. As shown in Table 1, the participants were matched for socioeconomic status and for height, weight and BMI. Hb and serum Fe levels were lower and serum Fe-binding capacity was higher in the anaemic participants than in their normal peers (Table 1). The dietary intakes of energy, carbohydrate, fibre and Fe were not significantly different (Table 2) between the two groups. Intakes of milk and milk products were low in both the groups, but were not significantly different.

Faecal levels of Bifidobacterium genus, Bacteroides–Prevotella group, E. rectale and C. leptum were similar between the two groups (Fig. 1). However, faecal levels of L. acidophilus group bacteria were significantly lower in anaemic women (median 6·60 £ 10$^{28}$, interquartile range 1·2 £ 10$^{-8}$–2·1 £ 10$^{-6}$) than in the normal group (2·95 £ 10$^{26}$, 3·7 £ 10$^{-7}$–2·1 £ 10$^{-5}$; $P=0·006$) (Fig. 2).

**Discussion**

The present study identified a significant reduction in the faecal population of lactobacilli in young women with Fe deficiency and anaemia in south India. Other major classes of bacteria in the stool were not significantly altered. Dietary ingestion of energy, total and insoluble dietary fibre, and milk products was similar in individuals with and without anaemia.

Mean dietary Fe intake in either group (8.8–8.9 mg/d) was in the range expected for this population, and was almost exclusively non-haem Fe$^{(5)}$. Certain lactobacilli have a growth requirement for Fe under specific environmental conditions$^{(3)}$. While it is possible that reduced faecal Lactobacillus numbers were secondary to low luminal Fe levels in the Fe-deficient women, there were no differences in Fe intake between the two study groups. It is therefore reasonable to speculate on other explanations for the isochronous occurrence of reduced Lactobacillus numbers and Fe-deficiency anaemia in these young women, and to consider the role of the colon in Fe absorption.

Approximately 10% of ingested Fe is absorbed in health, mainly or solely from the duodenum, aided by the presence of several Fe transporters in enterocytes. Evidence from experimental animals suggests that Fe may also be absorbed from the proximal colon. In pigs, the colon contributes to approximately 12% of Fe absorption$^{(14)}$. The Fe transporters, divalent metal transporter 1 and ferroportin, are expressed in the colon of animals and man$^{(14–17)}$, and expression may be increased in Fe deficiency states$^{(15,16)}$. In the proximal colon, the absorption of Fe and other divalent cations is enhanced by SCFA, which are produced by bacterial fermentation in the colon. Non-digestible disaccharides increased caecal SCFA pools and prevented Fe-deficiency anaemia in gastrectomised rats$^{(18,19)}$. Inulin, non-digestible in the small intestine, is extensively fermented in the proximal colon, producing large amounts of SCFA$^{(20)}$. Thus, a reduction in the faecal Lactobacillus population may contribute to reduced Fe absorption in the colon.

**Fig. 1.** Quantitative PCR of (a) Bifidobacterium genus, (b) Bacteroides–Prevotella–Porphyromonas, (c) Eubacterium rectale and (d) Clostridium leptum from faeces of anaemic and normohaemic young women, normalised to amplification of universal 16S ribosomal DNA. Bars depict median and interquartile range.

None of the differences was statistically significant.

**Fig. 2.** Quantitative PCR of Lactobacillus acidophilus group from faeces of anaemic and normohaemic young women, normalised to amplification of universal 16S ribosomal DNA. Bars depict median and interquartile range.

$P=0·001$ by unpaired t test after logarithmic transformation.
bowel, increased Fe retention in rats. Inulin also up-regulated mRNA expression for divalent metal transporter 1 and ferroportin, and increased faecal levels of *Lactobacillus* and *Bifidobacterium* species in pig colon. In the present study, dietary fibre intake (soluble and insoluble) was similar in anaemic and normal women. However, the dietary intake of specific non-digestible disaccharides and oligosaccharides was not quantified in the present study. Lactobacilli contribute to colonic fermentation, a process that reduces the inhibitory effect of phytate on Fe absorption. *L. acidophilus* has been shown to increase Fe uptake into Caco-2 cells in vitro by reducing total soluble phenols in the digesta. These variables (SCFA, phytate and phenols) were not investigated in the present study, and any comments pertaining to them must remain speculative.

In conclusion, the present study documents an interesting association between Fe deficiency and reduced faecal lactobacilli in young women in India. Understanding the relationship between Fe status and faecal lactobacilli has the potential to lead to alternative strategies to combat Fe deficiency in this population.

Acknowledgements

The authors have no conflicts of interest to declare. The study was supported by a research grant from the Christian Medical College, Vellore. R. B. was supported by a Senior Research Fellowship from the Indian Council of Medical Research. R. R. M. was partially supported by a Summer Fellowship of the Indian Academy of Sciences. The department received support through grant no. LSI-141/2002 (Funds for Infrastructure in Science and Technology, Government of India). R. B., R. R. M. and B. S. R. were responsible for conception of the study; R. R. M. was responsible for recruitment and follow-up of participants; R. B. and S. C. were responsible for the molecular analyses; H. J. and R. S. D. were responsible for dietary survey; B. S. R. was responsible for funding; and R. B. and B. S. R. were responsible for writing the manuscript.

References