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Research Paper

A Preliminary Clinical Evaluation of Probiotics *Pediococcus Acidilactici* MTCC5101 and *Bacillus Coagulans* MTCC492 on Young Anemic Women

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Abstract

Human intervention studies are a requisite to justify prophylactic and therapeutic effects of probiotics. A Lactic Acid Bacterial (LAB) isolate obtained from chilli pickle has been identified as *Pediococcus acidilactici* MTCC5101 based on 16S rRNA gene sequence. It is a bile tolerant strain which produces Pediocin CP2 with a wide antibacterial range. Present study is focused on evaluation of probiotic potential of *P. acidilactici* MTCC5101 and its colonization in human gastrointestinal (GI) tract. Further in a pilot scale pre-clinical trial among 30 young anemic women, role of *P. acidilactici* MTCC5101 along with another probiotic *Bacillus coagulans* MTCC492 was assessed in absorption of therapeutic iron salt in human GI tract.

In vitro adhesion assay of *P. acidilactici* MTCC5101 using Caco-2 cells confirmed the adherence of strain to intestinal epithelial cells. Results of *in vivo* analysis for molecular identification of strain in fecal samples of volunteers coincide with the *in vitro* findings. The strain was found to be capable of colonizing the GI tract after 4 weeks of oral consumption. Hemoglobin analysis of 4 week pilot study on anemic females showed that the supplementation of iron salt along with the probiotic mixture improved iron absorption in this group as compared to other two groups. Group III showed an average increase of 2.25±0.6 gm/dL in Hb level as compared to 1.56±0.9 gm/dl in Group I and 1.65±0.4 gm/dl in Group II. The present study confirmed the probiotic potential of *P. acidilactici* MTCC5101 and *Bacillus coagulans* MTCC492 with applications in food industry.

***Keywords*:** Lactic acid bacteria, Probiotics, Gastrointestinal tract, Anemia

Probiotics are live microbial feed supplements which beneficially affect the host by improving microbial balance in its gastrointestinal (GI) tract (Fuller, 1989). All Lactic acid bacteria (LAB) are considered as probiotics and Generally Recognized

as Safe (GRAS) due to a long history of safe use in foods. Studies have reported non-pathogenicity of most LAB strains in animals (Monose *et al.,* 1979; Donohue *et al.,* 1993) as well as humans (Saxelin *et al.,* 1996; Kailasapathy and Rybka, 1997). However, few reports on bacteremia due to consumption of probiotics are available in literature (Husni *et al.,* 1997; Arpi *et al.,* 2003; De Groote *et al.,* 2005; Land *et al.,* 2005). Over the years, probiotic bacteria have been used in the production of various dairy products and dietary supplements due to their GRAS status. They are commonly consumed as part of fermented foods with specially added active live cultures (FAO/WHO, 2002).

A Number charges of studies from the various research groups reported the health-promoting properties of LAB such as improving intestinal tract health, enhancing the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individuals and reducing risk of certain cancers which emphasize upon their consideration as probiotics (Hirayama *et al.,* 2000; Ouwehand *et al.,* 2002; Parvez *et al.,* 2006; Scholz-Ahrens *et al.,* 2007; Yeo *et al.,* 2010). The mechanism behind such health beneficial effects are largely unknown, but may involve modifying gut pH, antagonizing pathogens through production of antimicrobial compounds, competing for pathogen binding and receptor sites as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and production of lactase (Chichlowski *et al.,* 2007; Corcionivoschi *et al.,* 2009; Cullingen *et al.,* 2009; Ng *et al.,* 2009; Randhawa *et al.,* 2011; Randhawa *et al.,* 2012). For exerting the beneficial health effects on host, probiotics should be able to survive the passage through GI tract and overcome highly acidic environment of stomach, action of digestive enzymes and bile salts. They should also be capable of adhering to intestinal epithelial cells (Collado *et al.,* 2009, Balgir *et al.,* 2013).

Anemia occurs due to low iron absorption in digestive tract due to presence of inhibitory compounds such as phytates, oxalates and phosphates in food. Parasitic infestations such as Trichuris (whipworm) and hookworm can also lead to iron deficiency anemia, as infestation by these worms in large Number chargess lead to chronic intestinal blood loss (WHO, 1996; Crompton and Nesheim, 2002). Amongst the population, a sizable segment composed of adolescent girls, represents a practically vulnerable group at risk for iron-deficiency anemia because of maximum physical, psychological and behavioural changes during this phase of life and negligence of family towards girl child. Blood loss due to menstrual cycle poses an added burden towards the crisis (Siddharam *et al.,* 2011).

Three main strategies (alone or in combination) have been followed from time to time for correcting iron deficiency: Dietary modification to improve iron intake and bioavailability; Iron supplementation and iron fortification of foods. Iron supplementation can be targeted to high-risk groups such as pregnant women, and

can be cost effective, but the logistics of distribution and absence of compliance are major limitations. For oral supplementation, ferrous iron salts majorly ferrous sulphate, ferrous gluconate, ferrous oxalate and ferrous phosphate are preferred because of their low cost and high bioavailability. As a side effect, these salts cause irritation in gut when consumed on empty stomach. Hence, these salts should be consumed only after meals (Zimmerman and Hurrell, 2007).

To increase absorption of iron by intestinal cells, probiotics can be beneficial. They create an acidic environment in the intestinal tract which makes iron and other minerals more absorbable. A Number charges of clinical studies where probiotics have been incorporated in diet of test volunteers for a certain time period further strengthen the fact that probiotics influence iron absorption positively (Bering *et al.,* 2006). There are studies that provide evidence in favour of probiotic exerting a beneficial effect on mineral absorption like iron and calcium. The underlying mechanism may possibly be an enhanced absorption of these minerals from diet due to low pH or acidic conditions produced by probiotics bacteria. Low pH conditions lead to solubilization of minerals from food leading to an increased bioavailability in the gut. Degradation of mineral complexing phytic acid from food is another possible mode of action exerted by probiotics (Scholz-Ahrens *et al.,* 2007). Patel *et al.,* (2009) reported siderophore production in probiotics strains of Bacillus spp. Siderophores are low molecular mass (500–1000 Da) iron-chelating ligands which bind ferric ions with high affinity and are synthesized by microorganisms under iron-limited conditions. They solubilize the non-soluble surrounding iron and make it biologically available. Thus, making probiotics a part of diet plan can ameliorate anemic condition in human beings

Pediococcus acidilactici MTCC5101 is a probiotic strain capable of secreting a potent antibacterial bacteriocin Pediocin CP2 exhibiting antimicrobial activity against a wide range of Gram-positive, Gram-negative bacteria as well as fungi namely. The antimicrobial range of Pediocin CP2 includes a wide range of Gram-positive, Gram-negative bacteria as well as fungi i.e. *Listeria monocytogenes, Enterococcus faecalis, Pseudomonas putida, P. aeruginosa, Streptococcus mutans, Neisseria mucosa, Leuconostoc mesenteroides, Clostridium sporogenes, Pediococcus acidilactici LB42, P. pentosaceus, Lactobacillus brevis, Aspergillus* strains, etc. (Kaur and Balgir, 2004). Genes encoding production of Pediocin CP2 are localized to the ped operon present on plasmid pCP289 of *P. acidilactici* MTCC5101 (Kaur and Balgir, 2007; Kaur and Balgir, 2008). The characteristics such as acid resistance, bile tolerance, survival through gut and adhesion of the strain to intestinal cells strongly emphasize the probiotic potential of the strain and points towards its potential application as a prophylactic and therapeutic agent against pathogenic bacteria in gut. In a recent study *P. acidilactici* MTCC5101 was proven to efficiently adhere in vitro to intestinal epithelial cell line Caco2 in

culture and in vivo amongst healthy female volunteers, as well. The consumption of the probiotic strain was observed to improve hematological parameters of volunteers in wellness assays as well (Balgir *et al.,* 2013).

Bacillus coagulans is a spore forming probiotic strain which is capable of surviving in extreme temperatures, stomach acidity, digestive enzymes and bile salts (Keller *et al*., 2010). It has been used in functional food products for human consumption due to its role in ameliorating symptoms of various gastrointestinal disorders and as an immunomodulating agent in viral infections and sold under brand names Lactospore (Sabinsa Corp.), Lacbon and Lacris (Uni-Sankyo) and Lactopure (Pharmed Medicare). *Bacillus coagulans* has been mislabelled as *Lactobacillus sporogenes* on product labels (Sanders *et al*., 2001; Sanders *et al*., 2003). This misclassification has been amended and the microorganism has now been referred to as *Bacillus coagulans* in 5th edition of Bergey's Manual (1939). The present work is a controlled feeding trial involving anemic female volunteers who were fed *Pediococcus acidilactici* MTCC5101 and *Bacillus coagulans* MTCC492 supplemented iron capsules for one month. Hemoglobin of volunteers was analyzed before and one week after trial period.

Materials and Methods

Reagents

The following reagents were obtained from Himedia Chemical Pvt. Ltd., Bombay, India: de Man Rogosa and Sharpe (MRS) broth and Nutrient broth. *Pediococcus acidilactici* MTCC5101 was isolated in Genetic Engineering Lab, Department of Biotechnology, Punjabi University, Patiala (Kaur and Balgir, 2008). The culture was identified by MTCC, IMTECH, Chandigarh. *Bacillus coagulans* MTCC492 culture was procured from MTCC, IMTECH, Chandigarh. Ferrum Phos tablets used as source of iron were procured from Holistic Remedies Pvt. Ltd. and Bioforce AG Switerzerland.

Phylogenetic analysis of P. acidilactici MTCC5101 based on 16S rRNA gene sequence

16S rRNA gene was amplified via PCR using 16S rRNA gene specific primers: Forward Primer 5'-AGAGTTTGATCCTGGCTCAGG-3' and Reverse Primer 5'- GGAGGTGATCCAGCCGC-3'. Amplified PCR product was analysed qualitatively on 0.8% agarose gel. The sequence of the amplified gene was provided by Merck, Hyderabad. Bioinformatic analysis of the retrieved sequence was carried out using blastn and clustal omega for correct identification of the strain.

In vitro adhesion and in vivo survival study of P. acidilactici MTCC5101

For assessment of *in vitro* adhesion of probiotic strain *P. acidilactici* MTCC5101, enterocyte-like Caco-2 cells were used and adhesion assay was performed as described previously by Bernet *et al.* (1993). The specimens were examined with a JEOL JSM-6610LV SEM at Indian Institute of Technology, Ropar, Punjab, India. The Number charges of bacterial cells adhered to Caco-2 cells were counted on a Number charges of microscopic fields and the values were represented as average Number charges of bacterial cells adhered per 100 Caco-2 cells.

In vivo survival and persistence of *P. acidilactici* MTCC5101 in human GI tract was assessed as a controlled, parallel, 6 week trial, with 4 weeks of intervention and 2 weeks wash-out period. The study was carried out on 10 healthy volunteers who gave their consent to join the feeding trial. The study was approved by the Institutional Clinical Ethics Committee vide clearance no. ICEC/3/2011 and experimental work was carried out as per the guidelines of Indian Council of Medical Research for conducting research on human subjects (2006). Molecular identification of strain was carried out in fecal samples of volunteers fed fresh buttermilk (Verka Co. Ltd., Patiala, India) supplemented with *P. acidilactici* MTCC5101 (10^8–10^{10} cells/ml). Presence of *pedA* specific 323bp fragment was confirmed using 2% agarose gel (Balgir *et al*., 2013).

Clinical study on anemic subjects

The study was conducted at the Department of Biotechnology, Punjabi University, Patiala, as a controlled 4 weeks dose–response trial. Study involved feeding of live GRAS grade probiotic bacteria, *Pediococcus acidilactici* MTCC5101 and *Bacillus coagulans* MTCC492 to 30 adult female volunteers (19-22 years) for a period of one month. Hemoglobin (Hb) levels ≤ 10 was selected as the inclusion criteria for short listing volunteers. All participants gave their informed consent prior to the trial.

Morphological characteristics of probiotic cultures were examined using Gram staining (Gram, 1884). Biomass was obtained by growth in specific culture media, harvested using centrifugation and freeze-dried. Cell counts were made using haemocytometer (Mather *et al*., 1998). Ferrum Phos tablets were crushed and dispensed into capsules in powdered form according to prescribed dosage (400mg/day). Freeze-dried probiotic cultures of *P. acidilactici* MTCC5101 and *B. coagulans* MTCC492 were also dispensed into capsules as an equal mix of both strains (10^6 CFU/day of each). Capsules containing probiotic mixture along with Ferrum Phos were also prepared. Three age matched intervention groups, each having 10 members were prepared as follows:

GROUP I – Only Ferrum Phos

GROUP II – Only Probiotic mixture

GROUP III – Ferrum Phos + Probiotic mixture

Volunteers received a daily dosage of two capsules of either iron tablets (Group I) or probiotic mix (Group II) or both (Group III), respectively. They were instructed to take capsules after a meal (Montrose and Floch, 2005). Hb of volunteers was assessed before and one week after the trial period by method of Haldane (1901). At enrollment the participants received verbal information about the objectives of the study. Participants were followed regularly about changes in their well being during the trial at frequent intervals.

Results and Discussion

Morphological Examination of probiotic strains

Gram staining of bacterial cultures was performed and views were captured under microscope at different magnification. Gram stained *P. acidilactici* MTCC5101 were observed as Gram-positive cocci arranged in tetrads (Fig.1a) whereas B. *coagulans* MTCC492 appeared as Gram-positive rods (Fig. 1b).

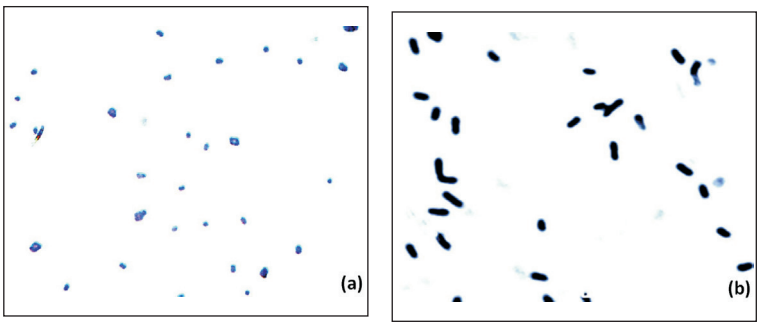

Fig. 1 Gram stained bacteria at 40X magnification, (a) P. acidilactici MTCC5101; (b) B. coagulans MTCC492

Phylogenetic analysis based on 16S rRNA gene sequence of P. acidilactici MTCC5101

Fig. 2 shows PCR amplified ~1.5kb 16S rRNA gene of *P. acidilactici* MTCC5101. A partial (524bp) 16S rRNA gene sequence obtained after sequencing of the product has been deposited in GenBank under accession no. KF305774. Homology analysis of partial 16S rRNA gene sequence (524bp) using blastn resulted in 103 sequence hits. Out of 103, 59 partial as well as complete 16S rRNA gene sequences of various *Pediococcus* strains were selected for phylogenetic analysis using Clustal omega. Phylogram was generated to calculate the evolutionary distances (sum of horizontal distances between two leaves) between different strains as is evident

from Fig. 3. Based on phylogram, two groups of *Pediococci* can be distinctly marked i.e. first comprising of strains of *P. pentosaceus* and second that of *P. acidilactici. P. acidilactici* MTCC5101 16S rRNA gene shows close resemblance to *P. acidilactici* strains and is evolutionary closer to second group. Thus, homology and evolutionary data points towards right direction as far as strain identification based on 16S rRNA genomic sequences is concerned.

Fig. 2. 1% agarose gel showing: Lane 1- Novagen Perfect DNA™ Marker (0.05-10kbp); Lane 2- 1.5kb 16S rRNA gene product

In Vitro Adhesion and *In Vivo* Persistence Study of *P. acidilactici* MTCC5101

SEM micrographs of in vitro adhesion assay of P. acidilactici MTCC5101 using Caco-2 cells clearly illustrate a very high tendency of the selected probiotics strain to adhere to intestinal epithelial Caco-2 cells. Photographs were captured at different microscopic fields and counts showed that at an average 152±33 cells adhered per 100 Caco-2 cells as shown in Fig. 4(a) and 4(b). Molecular analysis via pedA specific PCR of plasmids extracted from fecal samples of volunteers showed a gradual increase in 323bp amplicon concentration from 5th day to 30th day samples. The results clearly illustrate the ability of P. acidilactici MTCC5101 to persist and colonize in the human gut which further strengthens the probiotic properties of the strain (Balgir *et al.,* 2013).

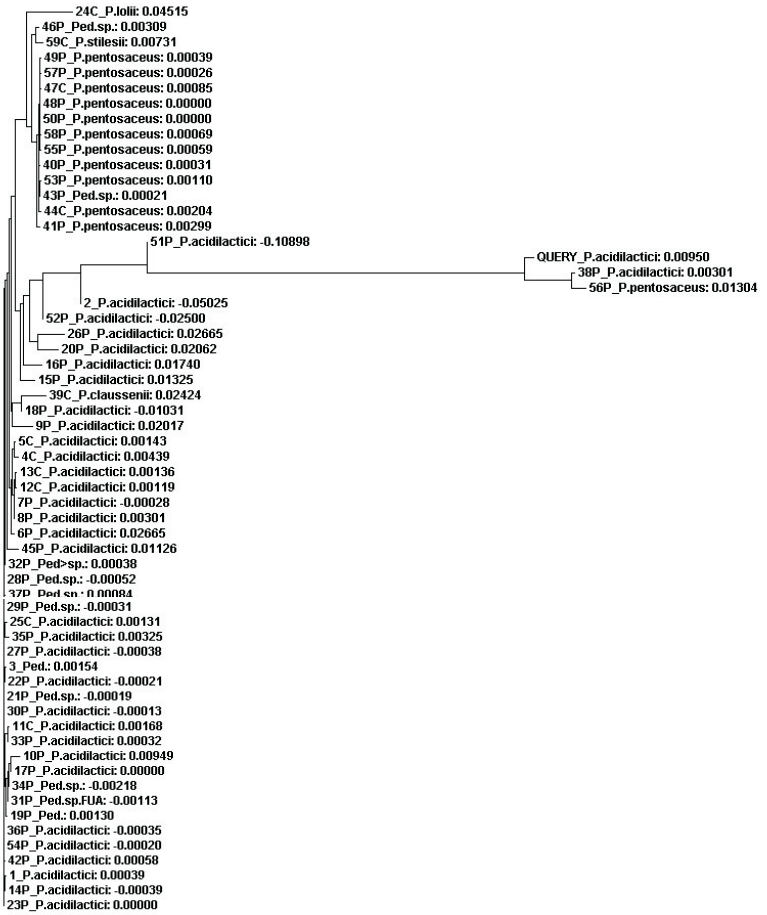

Fig. 3. Phylogram of 16S rRNA gene sequences showing evolutionary distances between different Pediococci

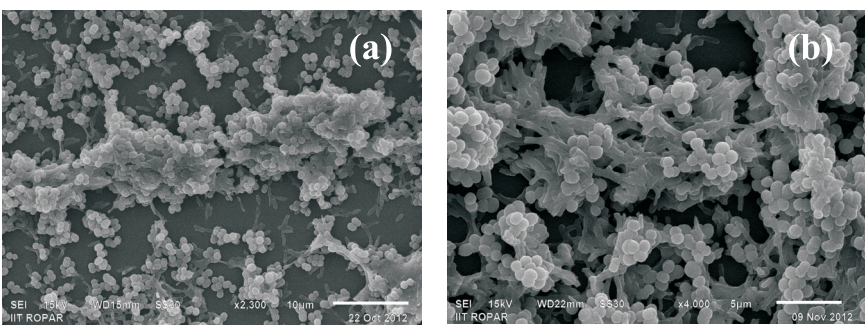

Fig. 4. SEM micrographs of P. *acidilactici* MTCC5101 adhered to Caco-2 cells: (a) at magnification 2,300x; (b) at magnification 4,000x

Hemoglobin (Hb) Estimation

Hb of volunteers was assessed one week before and one week after the trial period using hemometer. Data values for intervention groups are shown in Table 1. Fig. 5 shows a graphical representation of the same.

Table 1. Hemoglobin concentration of volunteers (gm/dl)

GROUP I			GROUP II			GROUP III		
Initial	**Final**	**Diff.**	**Initial**	**Final**	**Diff.**	**Initial**	**Final**	**Diff.**
10	10.8	0.8	8.0	10.0	2.0	8.5	10.5	2.0
8.8	11.0	2.2	7.0	8.8	1.8	9.6	12.0	2.4
8.0	11.8	3.8	10.0	11.5	1.5	9.8	Drop out	Drop Out
7.0	9.4	2.4	8.0	9.5	1.5	9.0	12.0	3.0*
9.5	10.5	1.0	9.0	10.0	1.0	9.0	10.0	1.0
9.5	10.5	1.0	9.0	10.5	1.5	6.5	9.6	3.1*
10.0	11.0	1.0	9.0	10.0	1.0	10.0	12.4	2.4
8.8	9.8	1.0	8.8	10.5	1.7	10.0	11.5	1.5
8.2	9.4	1.2	8.0	10.5	2.5	7.0	10.4	3.4*
10.2	11.4	1.2	8.0	10.0	2.0	10.0	11.5	1.5
9.0±1.0	10.56±0.8	1.56±0.9	8.48±0.8	10.13±0.7	1.65±0.4	8.94±1.2	11.03±0.9	2.25±0.6

-'*' represents maximum Hb values obtained in study
-Values in last row (in bold) represent mean±SD values of Hb concentration of respective groups

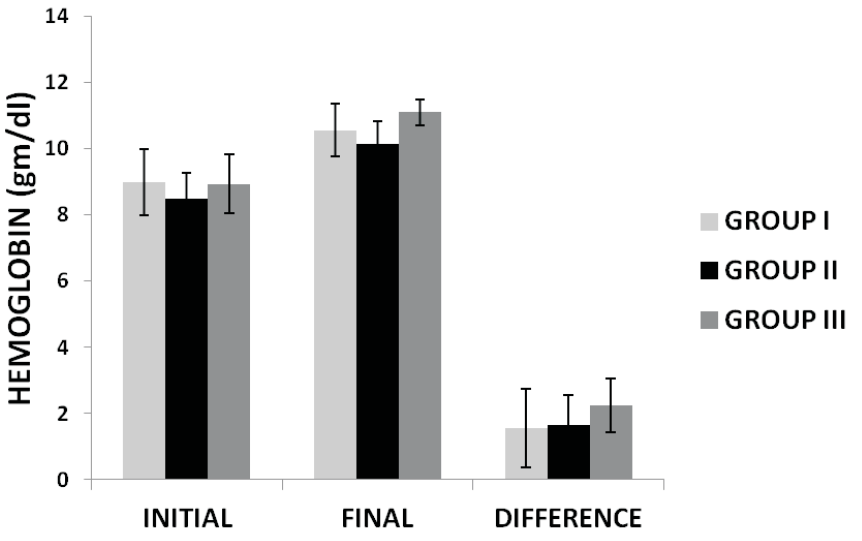

Fig. 5. Graphical representation of variation in hemoglobin concentration in intervention groups before and after the trial

Results and Discussion

Iron is one of the most frequently deficient nutrients in populations not just in the developing world but also in developed countries. The possible solution to such a major problem is not only to adopt an iron-rich diet, reason being low absorption of iron from food due to the presence of mineral absorption inhibitory factors. A benign way of supplementation keeping absorption in view is required. Studies have proven the efficacy of probiotics in improving iron bioavailability in gut of animals and humans (Bering *et al.,* 2006; Scholz-Ahrens *et al.,* 2007; Sazawal *et al.,* 2010).

The present study is based on a controlled oral feeding trial of a probiotics containing iron formulation being fed to 30 anemic adult female participants for one month. *Pediococcus acidilactici* MTCC5101 and *Bacillus coagulans* MTCC492 were the probiotic strains used in this study. Response of volunteers to iron intervention is measured using an indicator which shows the largest and most consistent change in response to iron uptake. Among hematological indicators related to iron storage, Hb is the most commonly used indicator since it is easy, less time consuming and inexpensive (Mei *et al.,* 2005).

Study revealed a noticeable increase in average Hb concentration (gm/dl) of group III as compared to the other two groups (Fig. 5). Group I consisted of volunteers fed on Ferrum Phos only. This group showed improvement in Hb levels (0.8 to 3.8 g/dl avg.= 1.56±0.9) after one month of supplementation with Ferrum Phos. Group II constituted volunteers who were fed probiotics mixture of both strains without iron salt. This group also showed improvement in Hb status (1.0 to 2.5g/dl , avg. = 1.65±0.4), which may be attributed to improvement in iron absorption. An overall health status was also observed to improve. As observed in the study of Bering *et al.,* (2006) wherein the effect of an oat gruel fermented with *Lactobacillus plantarum* 299v on non-heme Fe absorption from a low-Fe bioavailability meal was compared with a pasteurised, fermented oat gruel and non-fermented oat gruels was assessed on 24 healthy women. The fermented gruel increased non-haem Fe absorption from a phytate-rich meal in young women, indicating a specific effect of live *L. plantarum* 299v and not only an effect of the organic acids.

Another study by Sazawal *et al.,* (2010) has proven that overall health and growth parameters were observed to increase in pre-school children along with improvement in their iron deficiency after probiotic intake. 624 children received probiotic *Bifidobacterium lactis* HN019 and 2.4 g/day of prebiotic oligosaccharides fortified milk for a duration of 1 year. Results revealed that the consumption of *B. lactis* HN019 and prebiotic-fortified milk resulted in a smaller Number charges of iron-deficient preschoolers with increased weight gain.

Group III volunteers fed both probiotics mixed with the same amount of Ferrum Phos as given to Group I, the iron absorption was observed to be maximum (1.0-3.4 g/dl avg.= 2.25±0.6), pointing to beneficial effects of probiotics in case of anemic young girls as was hypothesized based on mechanism of iron absorption aided by probiotics (Scholz-Ahrens *et al.,* 2007; Patel *et al.,* 2009). Members of group III responded very well to the formulation as is evident from the results, showing an increase in Hb levels ranging from 1-2.4g/dl in majority and more than 3g/dl in 33.3% of the volunteers. The use of probiotics along with iron supplementation certainly improves the absorption of iron in humans and can aid in amelioration of iron-deficiency anemia among women. The study emphasizes the fact that the tested LAB strains are potent probiotics having significant effect on iron absorption in humans. This proves the concept of strain specific beneficial effects of probiotics.

Conclusion

The present study confirms the potential of *P. acidilactici* MTCC5101 and *Bacillus coagulans* MTCC492 as probiotic strains with applications in food fortification as well as therapeutics for improved iron absorption. Further study to identify other such probiotic strains for specific anemia ameliorating affects need to be investigated.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgements

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