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Introduction

―Almost all our health concerns can be traced back to our belly. Ensure a healthy gut and the rest will take care of itself.‖

- **Behzad Azargoshasb (2018)**

Iron is a vital micronutrient in the human health system. In several developing countries like India, Iron Deficiency Anaemia (IDA) poses a notable public health challenge, particularly affecting impoverished regions, socioeconomically disadvantaged communities, and children born to women lacking formal education (Bhatnagar *et al.,* 2021). The societal and economic impact of IDA on developing nations is substantial, with estimated productivity losses amounting to 1.3% of India's Gross Domestic Product (FAO 2021). Recent inquiries have yielded several valuable insights concerning its intrinsic significance to a range of critical physiological mechanisms, encompassing DNA synthesis, oxygen transport and energy production (Roemhild *et al.,* 2021). Hence, Iron bioavailability is a crucial concept of functional relevance in maintaining nutritional balance. It refers to the proportion of ingested iron that is absorbed and subsequently made available for physiological utilization. Iron absorption is a multifaceted process characterized by a sequence of precisely orchestrated stages, including the solubilization of iron compounds derived from the diet, the conversion of ferric iron to its ferrous form, the active transportation of iron across the intestinal epithelium, and the eventual storage within cellular reservoirs (Li *et al.,* 2021). Numerous initiatives have been launched to address IDA in India; however, their effectiveness has been hindered by administrative irregularities, gaps in program implementation, limited medical coverage, and societal unawareness rooted in cultural practices and beliefs (Sharma *et al.,* 2003). Moreover, there is a tendency to avoid designing interventions that address the multifaceted nature of the issue.

To combat IDA in India, strategies involving dietary approaches to enhance iron bioavailability have been suggested. Nevertheless, it is crucial to acknowledge that India continues to harbour a significant portion of the global undernourished population, with over

190 million people affected, exacerbated by the fact that around one-fourth of the world's chronically undernourished individuals reside in India (FAO 2017). This situation is exacerbated by the diminishing contributions of agriculture to India's Gross Domestic Product from 1951 to 2011 and a persistently high poverty rate, reaching nearly 30 percent (Pernet *et al.,* 2019). It can be stated that exploring nutrition-centred strategies to enhance dietary iron absorption and bioavailability is crucial, which explains the need for the development of food products that are economical and accessible.

The classification of dietary iron encompasses two primary forms: heme and non-heme iron. The non-heme iron variant is prevalent in a spectrum of dietary sources, encompassing both plant-derived and animal-derived origins. In contrast, heme iron is uniquely restricted to animal-derived food sources, notably encompassing meat, fish, poultry, and eggs (Kumar *et al.,* 2022). Notably, heme iron is distinguished by its superior bioavailability, a characteristic attributed to its efficient absorption mechanism, which obviates the requirement for co-factors to facilitate its uptake. On the contrary, non-heme iron, which holds significance for individuals adhering to vegetarian diets, exhibits a comparatively lower bioavailability due to its susceptibility to the influence of dietary enhancers, inhibitors, and the existing iron stores within the body. Approximately 25% of dietary heme iron undergoes absorption, in contrast to roughly 17% of non-heme iron. The bioavailability rates range from 14% to 18% in mixed diets, whereas in vegetarian diets, the range lies between 5% and 12%. Heme iron constitutes 6-9% of Indian dietary intake and contributes up to 30% of absorbed iron due to its enhanced bioavailability. On the other hand, dietary elements have a significant impact on the bioavailability of iron, with some known to increase iron absorption and others to decrease it (Abbaspour *et al.,* 2014).

Ascorbic acid, predominantly found in fruits and vegetables, and moderate to sparingly in heme-rich sources such as meat, fish, and poultry is known to enhance iron absorption. On the contrary, certain components in our diet possess inhibitory qualities. Polyphenols and phytates, commonly found in plant-based foods, along with calcium typically found in dairy products, are known to decrease the absorption of iron. The complex structure of plant cell walls also plays a role in affecting the availability of minerals. These cell walls act as physical impediments that hinder the accessibility of iron compounds. Moreover, culinary techniques and cooking processes exert a substantial influence on the alteration of the availability of iron. They reduce the presence of antinutrients and facilitate the release of iron that is bound to proteins when the food's structure softens due to the application of heat. Hence, there is an impending need to develop and standardize food products that enhance iron bioavailability, especially via the non-heme form, by encompassing broader categories like vegetarianism and lactose intolerance. Pertaining to the need, recent studies have strategized on developing foods and beverages with certain combinations of ingredients, to enhance non-heme iron bioavailability.

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Marco et al., 2021). The pivotal role of gut microbiota in maintaining health and preventing a multitude of diseases is extensively studied over decades. The intricate interplay between these beneficial microorganisms and the host has been investigated, revealing their profound impact on various physiological processes, immunomodulation, and disease prevention. The utilization of Gram-positive lactic acid bacteria exhibiting anaerobic characteristics, absence of spore formation, and acid tolerance has gained prominence in the domain of food product innovation. These microorganisms, widely acknowledged for their Generally Recognized as Safe (GRAS) status, impart multifaceted benefits to food systems. Moreover, they play a role in improving the taste, texture, and nutritional content of food, while also speeding up the natural acidification process that occurs in food, as highlighted in a study by Mathur *et al.,* 2020. Probiotics like *Lactobacilli*, and *Bifidobacterium*, were found to influence iron bioavailability through the production of lactic acid (Rusu *et al.,* 2020). A clinical trial by Manoppo *et al.,* 2019 showed that vegetables and cereals fermented by *Lactobacillus* cultures significantly enhanced absorption of non-heme iron derived from a meal with inherently limited bioavailability of iron in humans. Probiotics aid iron absorption by converting it into accessible forms and producing related metabolites, highlighting their potential to enhance iron metabolism (Zakrzewska *et al.,* 2022 and Ciont *et al.,* 2023). *L. reuteri* is a specific strain of probiotic that has been extensively studied for its role in gut health and nutrient absorption (Mu *et al.,* 2018). Research has shown that *L. reuteri* can improve gut barrier function, reduce inflammation, and enhance nutrient absorption. These beneficial effects of *L. reuteri* on gut health make it a promising candidate for promoting nutrient uptake from seaweed and other dietary sources. Furthermore, A review study indicated that *L. reuteri* can enhance the absorption of iron, calcium, magnesium, and other essential minerals, thus addressing their deficiencies efficiently (Dinu *et al.,*2022), (Peluzio *et al.,* 2021). In a quasi-experimental study conducted in Indonesia, researchers investigated the effects of *L.reuteri* DSM 17938 on the absorption of iron in anaemic children (Manoppo *et al.,* 2019). The study employed pre- and post-intervention tests, using a control group. Similarly, a meta-analysis of eight studies examined the impact of the probiotic *Lactobacillus plantarum* 299v (Lp299v) on iron absorption, revealing a significant positive effect (Mu *et al.,* 2019). Furthermore, a case report demonstrated the use of *Lactobacillus reuteri* DSMZ17648 and heme iron supplements in managing severe, refractory anaemia associated with *Helicobacter pylori* infection (Hinduja *et al.,* 2023). *In vitro* faecal batch culture studies have demonstrated the fermentability of seaweed fibre constituents, thereby influencing the intricate interplay of colonic microbiota. This underscores the potential of seaweed consumption to exert a discernible impact on the dynamics of gut microbial communities, as reported by the research (Cherry *et al.,* 2019). The consumption of seaweed and the use of probiotics like *L. reuteri* can potentially contribute to improved gut health and enhanced nutrient absorption by modulating the gut microbiota (Skrypnik *et al.,* 2019).

Seaweeds are a diverse group of marine plants that have gained attention for their potential as a source of iron and a valuable source of nutrients, including proteins, antioxidants, and other nutrients (Gomez-Zavaglia *et al.,* 2019). Additionally, several underexploited and edible seaweeds were extensively studied for their nutrient bioavailability and role in promoting gut health. Zang *et al.,* 2023 have investigated that the constituents inherent in seaweed exhibit the potential to serve as prebiotic agents, exerting a favourable modulatory impact on the gut microbiota. Prebiotics are indigestible substances that support the proliferation of beneficial bacteria within the gastrointestinal tract. Seaweed components have been found to enhance bacterial populations in the gut, leading to a more diverse and balanced microbiota (Shannon *et al.,* 2022). This modulation of the gut microbiota has been associated with improved gut health and overall well-being (Lopez *et al.,* 2023).

The taxonomy of seaweeds delineates them into three primary categories: Chlorophyceae (green algae), Phaeophyceae or Heterokontophyceae (brown algae), and Rhodophyceae (red algae). This classification is predicated upon the constituents of their photosynthetic pigments, their observable colour spectra, and the nature of their stored nutritional reserves. Within the Indian context, the geographic landscape boasts a notable array of distinct ecological habitats. Specifically, this encompasses a noteworthy count of 97 major estuaries, 34 expansive lagoons, 31 significant mangroove ecosystems, 5 flourishing coral reefs, and 31 demarcated Marine Protected Areas (MPAs) (Titlyanov *et al.,* 2016).

Ulva lactuca, commonly known as sea lettuce, has garnered research interest for its potential as a source of bioavailable iron. Its higher bioavailability and affinity for iron and manganese compared to other metals were studied by (Malhotra *et al.,* 2023). Extracts from *Ulva lactuca* were used to synthesize iron nanoparticles, exhibiting bactericidal activity against enteropathogens (Bensy *et al.,* 2022). Among marine algae, *Ulva sp.* demonstrated significant bioavailable iron and ascorbic acid content (Garcia *et al.,* 2007). Evaluation of iron bioavailability, polyphenols, and antioxidants in three marine algae species highlighted *Ulva's* prominent iron bioavailability (Zhong *et al.,* 2020). Furthermore, *Ulva lactuca* was investigated for bioremediation potential and adeptness in removing heavy metals from water (Rahhou *et al.,* 2023).

The presence of phytochemical constituents within seaweeds adds an additional layer of complexity to iron bioavailability. Seaweeds encompass an array of chemical compounds like phytates, oxalates, tannins, and polyphenols, each with the potential to impede the absorption of iron. Also, minerals such as calcium, magnesium, zinc, and copper can competitively bind to ligands within the seaweed matrix, forming complexes that can either enhance or hinder iron bioavailability, depending on the type and concentration of minerals present (Peñalver *et al.,* 2020). These interactions are complex and can be influenced by various factors, including pH, redox potential, and the presence of chelating agents within the seaweed matrix (Oura *et al.,* 2023). Hence, nutrient-specific food combinations and the adoption of fermentation could be a more conventional approach for enhancing the bioavailability of a particular nutrient as well as efficiently reducing the anti-nutrient factors pertaining to the hindrance, respectively.

Safety assessment of edible seaweeds is of paramount importance to eliminate antinutritional factors hindering nutrient bioavailability (Barbaro *et al.,* 2022). Various techniques ensure edible seaweed safety, focusing on *Ulva lactuca*. Common methods include drying, thereby diminishing microbial risks via reduced water activity (del Olmo *et al.,* 2020). Yet,drying processes and handling can alter microbial quality, warranting attention when assessing seaweed safety (Lytou *et al.,* 2021). High-pressure processing effectively preserves multiple seaweeds, including *Ulva lactuca*, while retaining attributes like colour, texture, and antioxidants (WHO 2022). Culinary methods also influence *Ulva lactuca*, affecting its composition and properties based on cooking style and duration (Bayomy *et al.,* 2022). Heat treatments like boiling, steaming, and microwaving minimally impact toxic metal levels (Filippini *et al.,* 2021). Awareness of chemical, physical, and microbial hazards along the seaweed supply chain remains pivotal in upholding safety (Banach *et al.,* 2020).

The matrix of *Ulva lactuca* is composed of a myriad of compounds such as polysaccharides, proteins, polyphenols, and phytates, which significantly influence the release and availability of iron for absorption. Polysaccharides can form gels or matrices that encapsulate iron, potentially impeding its release and bioavailability (Ganesan *et al.,* 2019). On the other hand, certain compounds within the matrix, such as polyphenols, may act as chelating agents, enhancing iron solubility and absorption (Farghali *et al.,* 2023). The overall composition and structure of the matrix, which can vary among seaweed species and even within different parts of the same seaweed, exert a considerable influence on the fate of iron during digestion and subsequent absorption. *In vitro*, *in vivo* and *in silico* experimentation are usually done to quantify the bioaccessibility of nutrients at large.

Caco-2 cells are extensively used in *in vitro* bio-accessibility studies to assess the impact of dietary components on the absorption of non-heme iron from different food combinations in *in vitro* set-up systems (Shannon *et al.,* 2021). These cells, derived from colon carcinoma, possess an inherent ability to undergo spontaneous differentiation under conventional culture conditions. This differentiation process is particularly pronounced when the cells are cultivated within bicameral chambers, resulting in their transformation into entities resembling enterocytes and closely mirroring the characteristics of the human intestinal epithelium. This orchestrated differentiation provides researchers with a platform to explore nutrient assimilation, specifically iron, in a manner that closely approximates physiological conditions.

One of the central utilities of Caco-2 cells lies in their capacity to assess the transfer of iron across their monolayers in an apical-to-basolateral direction. This analytical approach equips the researchers to gauge the efficiency of iron bioavailability from different sources, such as ferric lactoferrin and iron citrate (Fonseca *et al.,* 2021). Notably, investigations have revealed a higher propensity for iron transfer from ferric lactoferrin when compared to iron citrate, while minimal transport events are observed with Fe-transferrin.

The significance of Caco-2 cells extends to their pivotal role as an instrumental tool for dissecting nutrient uptake and transference within the intricate milieu of the intestinal epithelium. Their widespread utilization has culminated in the elucidation of crucial aspects pertinent to nutrient bioavailability, with a particular focus on iron. Profound insights into the behaviour of Caco-2 cells under the influence of disparate nutrients afford researchers a deeper comprehension of the intricate mechanisms underpinning nutrient absorption and utilization within the human physiological framework (Lopez *et al.,* 2022).

The adoption of the colon carcinoma cell model as an *in vitro* paradigm offers discernible advantages over *in vivo* investigations. The controlled and manipulable environment inherent to *in vitro* models augments the ability to ascertain the relative contributions of diverse factors that modulate nutrient assimilation dynamics (Flores *et al.,* 2015). Furthermore, the consistent and reproducible nature of Caco-2 cells as a model system facilitates the systematic scrutiny of nutrient bioavailability (Liu *et al.,* 2022). Hence, researchers can acquire invaluable insights into the nuances of nutrient assimilation mechanisms and, thereby, make informed determinations concerning the bioavailability of nutrients within the context of consumable items and supplementary interventions.

The intricate process of uptake and transportation of iron in Caco-2 cells involves a complex interplay of various transporters and regulatory proteins. A prominent player in iron uptake within Caco-2 cells is the Divalent Metal Transporter 1 (DMT1). This transporter facilitates the translocation of Fe^{2+} iron across the apical membrane of enterocytes. Notably, the expression of DMT1 is tightly regulated in response to intracellular iron levels, with lower iron concentrations leading to an upregulation of DMT1 expression. Additionally, the transferrin receptor 1 (TfR1) assumes a crucial role by facilitating the binding and internalization of iron complexed with transferrin (Han *et al.,* 2023).

Complementing the role of transporters, a cohort of regulatory proteins exerts significant influence over the uptake and transportation of iron in Caco-2 cells. An illustrative example is Hepcidin, a potent negative regulator of iron absorption. Hepcidin orchestrates the

degradation and internalization of ferroprotein, a protein pivotal for iron export. Another regulatory protagonist is HFE, which plays a role in modulating the intrinsic iron status of small intestinal crypt cells and subsequently shaping iron absorption mediated by DMT1 in the mature enterocyte.

Emergent insights have underscored the impact of the direction of iron supply, whether via the basolateral or apical route, on the dynamics of iron transport within Caco-2 cells. Furthermore, transcytosis has been proposed as a contributory mechanism facilitating iron transport across the enterocyte. From the above studies, it is evident that the interplay of factors such as iron supply direction and potential participation of transcytosis further amplifies the complexity of iron transport across the enterocyte.

The information pertaining to the synergistic effects of seaweed and probiotics on iron bioavailability in Caco-2 cells is currently limited, which necessitates the need for this study. A study by Au *et al.,* (2000) detailed the utilization of the Caco-2 cell model in conjunction with extrinsic radio-iron. Their objective was to analyse the impact of enhancers, notably ascorbic acid, and inhibitors such as bran, phytate, and tea, on the bioavailability of iron. These components were incorporated into a semi-purified meal wherein egg albumen served as the primary protein source. The findings from this investigation highlighted two main aspects: firstly, the efficacy of Caco-2 cells as a tool for evaluating human iron absorption; and secondly, the feasibility of utilizing this cellular model to scrutinize the iron bioavailability resulting from diverse food combinations. Concurrently, Glahn *et al.,* (2009) undertook a study that encompassed the comprehensive evolution, validation, and application of the widely employed Caco-2 cell culture model. This model occupies a central role in the realm of elucidating the bioavailability of various nutrients.

Studies have confirmed that probiotics can control how genes are expressed and strengthen the gut barrier, especially in Caco-2 cells (Eady *et al.,* 2015). For instance, Wang *et al.,* (2022) have found that certain bacteria from chickens can prevent harmful bacteria from sticking to Caco-2 cells. Similarly, Han *et al.,* (2023) showed that a mix of different probiotic strains can improve the gut barrier's function by helping the cells respond to inflammation and making their connections tighter, when exposed to specific molecules. Furthermore, Behbahani *et al.,* (2019) discovered that a type of *Lactobacillus* called L15 can stop

Escherichia coli from attaching to Caco-2 cells. Another study by Wang *et al.,* (2022) used Caco-2 cells to investigate the strength of both the large intestine and small intestine barriers. They found that adding probiotics, especially strains of *Lactobacillus*, to the cells made them better at resisting the passage of molecules (measured as transepithelial electrical resistance, TEER). However, when the same was done with human intestinal cells (HIEC), they didn't show the same increase in resistance (Rooj *et al.,* 2010). This suggests that using Caco-2 cells is a suitable way to study how probiotics affect the gut barrier. The synergistic interaction between probiotics and Caco-2 cells summarises that probiotics can control genes and improve the gut barrier's function (FAO 2022).

The study entitled **"Development and Evaluation of *Ulva Lactuca* based Probiotic Beverage and *in vitro* Bioavailability of Iron using Caco-2 Cell Model"** aimed to study the iron bioavailability of seaweed incorporated probiotics using an *in vitro* Caco-2 cell model.

HYPOTHESIS OF THE STUDY:

- H_0 - Iron bioavailability will not be improved by the presence of *Ulva lactuca* in the probiotic beverage.
- H_0 - There is no enhancement in iron bioavailability between seaweed-incorporated probiotics and a standard iron supplement in the in-vitro Caco-2 cell model.

OBJECTIVES OF THE STUDY:

Primary Objective:

To study the *in vitro* iron bioavailability of seaweed-incorporated probiotic beverage using the Caco-2 Cell Model and to determine whether the enhanced iron bioavailability shall serve as a natural food alternative for Iron Deficiency Anaemia.

Secondary objectives: To

- Study the Nutrient, and Heavy metal composition of the selected seaweeds.
- Formulate and standardize the Seaweed-incorporated Probiotic Beverage.
- Determine the Nutrient profile, Probiotic potential, and Shelf life of the developed beverage.
- Assess the *in vitro* bioavailability of iron from the selected seaweed and developed beverage using Caco-2 cell models.

The following were the key highlights of the study:

- The iron bioavailability of seaweed-incorporated probiotics in the presence of Ascorbic acid, was considerably higher than that of seaweed based beverage.

- The iron bioavailability of seaweed-incorporated probiotics was almost comparable to that of ferrous sulphate, a commonly used iron supplement.

IDENTIFYING THE RESEARCH GAP

More research is needed to determine optimal probiotic strains and dosages that would pave the way for efficacious therapeutic applications.

- Identification of gaps in current knowledge and areas for further research pertaining to the association between probiotics, edible seaweeds, and iron bioavailability.
- Development of novel food products with ingredients that are nutrient dense, which is easy to prepare and consume.

Despite the potential beneficial effects of seaweed and probiotics on iron bioavailability, there is limited research on the association between these two factors. While previous studies have investigated the iron bioavailability of seaweed and probiotics separately, this study is one of the few to investigate the iron bioavailability of seaweed-incorporated probiotics using an *in vitro* Caco-2 cell model. This study aimed to identify and bridge the gaps in current knowledge regarding the association between probiotics, edible seaweeds, and iron bioavailability for further research.

To further address the gaps in the current study, the future scope of research should be extended to the following areas:

- The association between probiotics, edible seaweeds, and iron bioavailability.
- The correlation between the bio-accessibility of other micronutrients and their impact on iron bioavailability.
- Discussion of the need for *in vivo* studies and clinical trials to validate the findings from *in vitro* Caco-2 cell models.