3

Methodology

The methodology followed in pursuing the research study entitled **"Development and Evaluation of *Ulva Lactuca* based Probiotic Beverage and *in vitro* Bioavailability of Iron using Caco-2 Cell Model"** are elucidated under the following headings:

PHASE I

3.1 Selection, Nutrient, and Heavy Metal Analysis of the Selected Seaweeds.

3.1.1 Selection of Underexploited Edible Seaweeds from the Geographical Location.

3.1.2 Macronutrients and Micronutrients Analysis, Heavy Metal Analysis of Selected Edible Seaweeds.

i. Macronutrient Profile.

ii. Micronutrient Profile.

iii. Heavy metal Profile.

PHASE II

3.2 Development of the Seaweed-incorporated Probiotic Beverage.

3.2.1 Procurement and Processing of Raw Materials.

3.2.2 Formulation and Standardization of the Seaweed-incorporated Probiotic Beverage.

3.2.3 Sensory Evaluation of the Developed Beverage using a 9-point Hedonic Scale.

PHASE III

3.3 Determination of Physical Characteristics, Nutrient, and Nutraceutical Potentials of the Developed Beverage.

3.3.1 Assessment of Physical Characteristics of the Developed Beverage.

3.3.2 Assessment of Nutritional and Nutraceutical Profile of the Developed Beverage.

i. Estimation of Proximate Nutritional Composition of the Developed Beverage.

ii. Assessment of Qualitative and Quantitative Phytochemical Profile.

iii. Determination of *In vitro* Antioxidant Potential of the Developed Beverage.

a. DPPH Radical Scavenging Assay.

b. Ferric Reducing Antioxidant Potential (FRAP).

iv. Bioactive Compounds Profile using GC-MS/MS.

a. *In silico* ADME Profile of abundant Bioactive Compounds.

PHASE IV

3.4 Assessment of *in vitro* Probiotic Potential, Safety Assessment and Antimicrobial Activity of the Lactic Acid Bacteria in the Beverage.

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iv. Pancreatin Tolerance.

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vii. Glucose Fermentation.

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3.4.3 Safety Assessment and Antimicrobial Activity.

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iii. Antagonistic Activity of the Extracted Antibacterial Agents and Calculation of Minimum Inhibition Concentration (MIC).

3.4.4 Determination of Antimicrobial Activity, Storage and Shelf-life Study.

PHASE V

3.5 Assessment of *in vitro* Bioavailability of Iron from the Selected Seaweed and Developed Beverage using the Caco-2 Cell Model.

3.5.1 *In vitro* Simulated Digestion.

i. Dialysis.

3.5.2 Cell Culture.

3.5.3 Cytotoxicity Study on the Caco-2 cell Model.

3.5.4 Iron Uptake Study in Caco-2 Cell Model.

PHASE VI

3.6 Statistical Analysis of the Study Outcome.

3.7 Conceptual Framework of the Study.

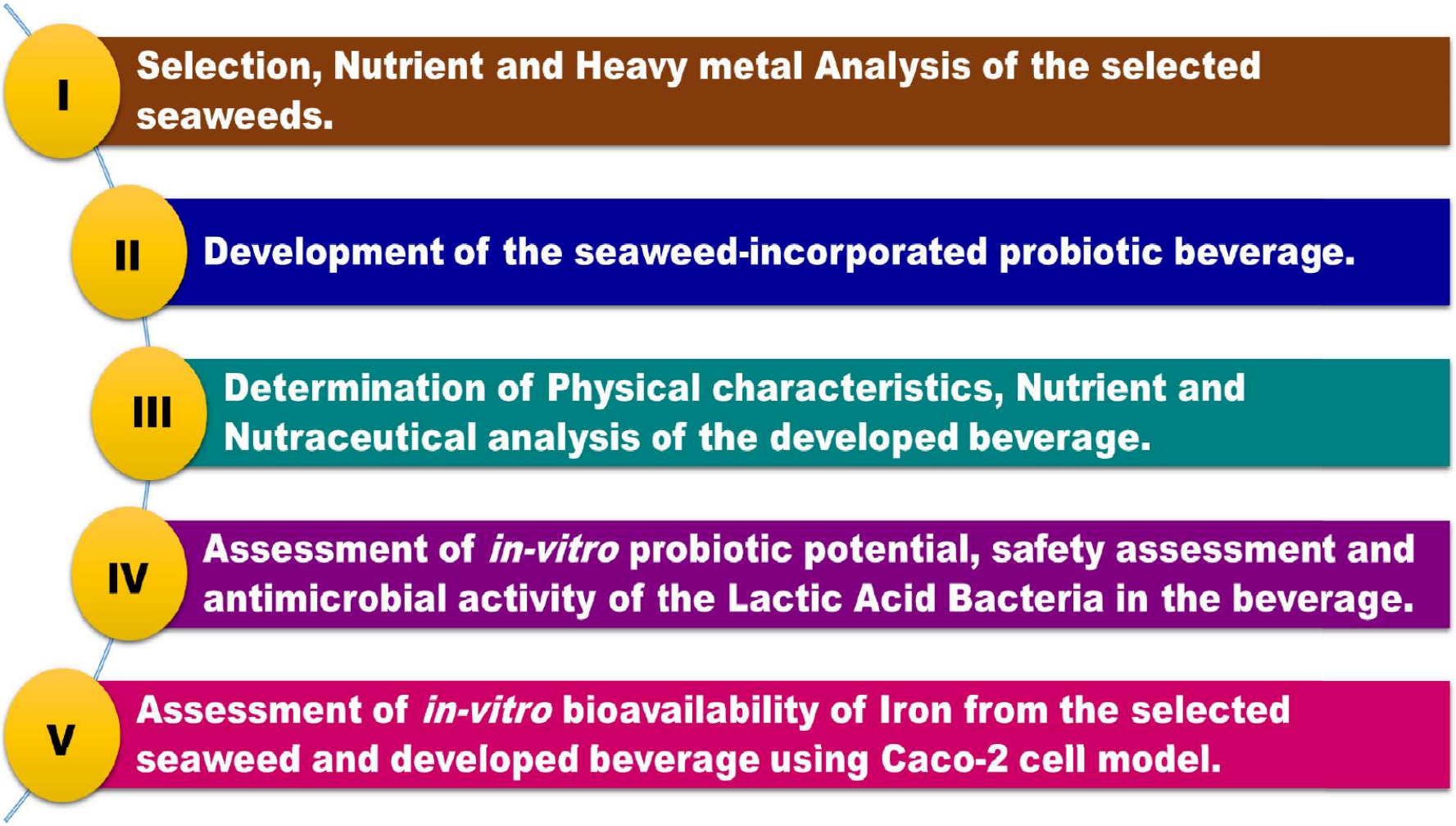

Fig. 3.1. Methodology of the Study.

The research study was approved by the Institutional Human Ethical Committee of the Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore (AUW/IHEC/FSN-19-20/XPD-37) which is depicted in the Appendix I. The conceptual framework of the study and the research design depiction is given in Figures 3.1 and 3.2.

PHASE I

3.1 Selection, Nutrient, and Heavy Metal Analysis of the Selected Seaweeds.

3.1.1 Selection of Underexploited Edible Seaweeds from the Geographical Location.

Ramanathapuram District in Tamil Nadu was selected as the locale for the study. Gulf of Mannar with all 21 islands along the 140 Km stretch between Tuticorin and Rameswaram (Lat. 8° 55‖ – 9° 15‖ N and Long. 78° 0‖ – 79° 16‖ E) has been rightly considered for a Marine Biosphere Reserve. The Gulf of Mannar coast covers Tuticorin, Tirunelveli, and a portion of Kanyakumari and Ramanathapuram Districts stretching from Kanyakumari in the southern end of the Indian Peninsula to Pamban in the north. The Indian coasts have a rich biodiversity of marine algae with about 850 recorded species. As the seacoast of the Gulf of Mannar and Palk Bay of Ramanathapuram District contains a rich vegetation of marine algae and the use of seaweeds as food is not quite popular in India, the present investigation has been chosen to use iron-rich edible seaweed as a potential prebiotic and as a source to complement probiotics to enhance iron bioavailability of the food product. Consumption patterns of several underexploited edible seaweeds in the area have been extensively studied and further analyzed for their nutritional properties (Abirami and Kowsalya *et al.,* 2012). The taxonomy of seaweeds delineates them into three primary categories: Chlorophyceae (green algae), Phaeophyceae or Heterokontophyceae (brown algae), and Rhodophyceae (red algae) (Titlyanov *et al.,* 2016). Four edible underexploited seaweeds selected for the study include *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis,* and *Sargassum polycystum*. Both the *Ulva* species (*spp.*) belong to the Chlorophyceae category whereas *Gracilaria spp.* belongs to Rhodophyceae and *Sargassum spp.* belongs to the Heterokontophyceae categories respectively. The availability of seaweeds throughout the year can be categorized as pre-monsoon (June-September), monsoon (October-January), and post-monsoon (February-May) seasons. *U. lactuca* and *U. reticulata* are less abundant in pre-monsoon and monsoon but are most abundant in post-monsoon seasons. *S. polycystum* is sparse in both pre-monsoon and monsoon seasons but is available sparsely in post-monsoon seasons. *G. edulis* is sparsely

found in all the seasons throughout the year (Mary Josephine *et al.,* 2013). The selected seaweed species are edible seaweeds consumed occasionally by the local population. The seaweeds were identified and verified with the help of Central Marine Fisheries Research Institute CMFRI bulletin No.41. Figure 3.2 depicts the locale of the seaweed selection.

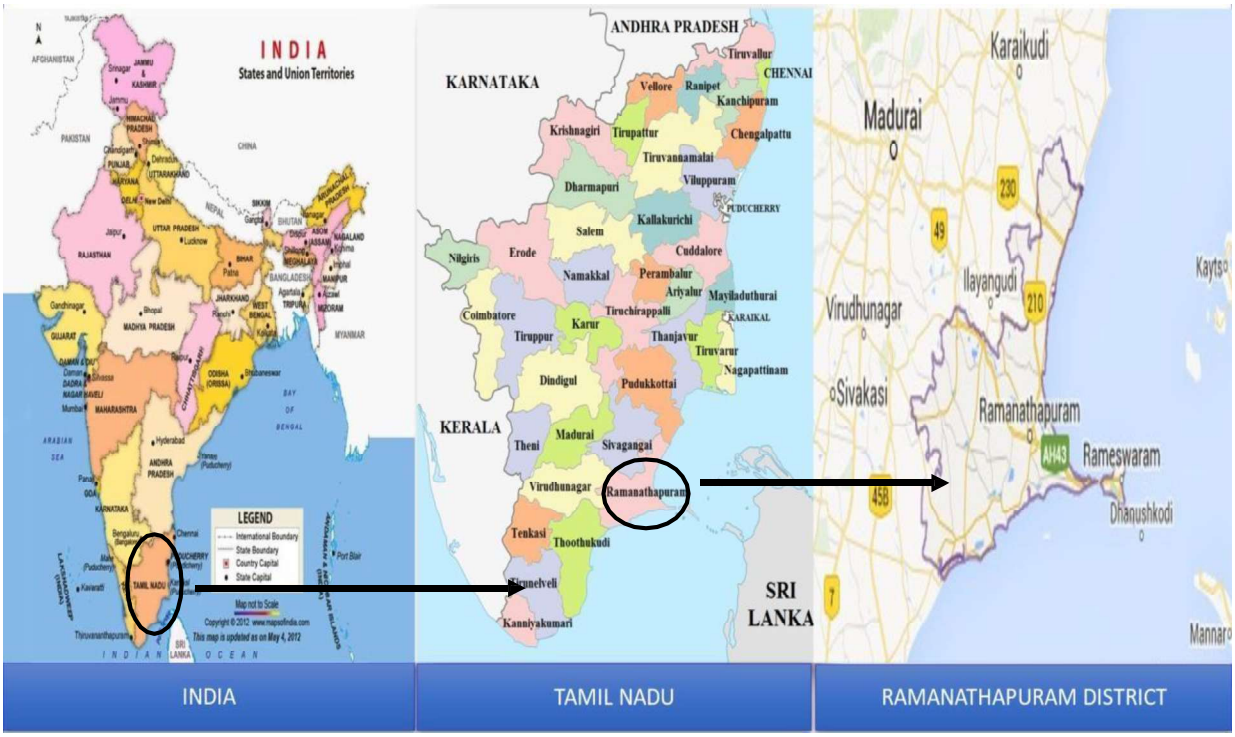

Fig 3.2. Locale Of The Seaweed Selection

3.1.2 Macronutrients and Micronutrients Analysis, Heavy Metal Analysis of Selected Edible Seaweeds.

A solution of 5 -10 % formaldehyde in seawater was prepared to preserve the seaweed samples as per the wet preservation method suggested by Dhargalkar, (2004), from the collection site until the sample is prepared for investigation.

Sample Preparation

Seaweeds were collected fresh with the help of sea divers trained in handpicking these seaweeds. The collected seaweeds were washed in seawater to remove all the extraneous matter such as epiphytes, shells, associated fauna, and adhering sand particles in the collected area and packed in aseptic bags. They were further cleaned with fresh water to remove extraneous matter such as epiphytes, sand particles, pebbles, and shells. These seaweeds were then washed thoroughly to ensure that all the dirt was removed and spread out at room temperature for drying for a period of 24-48 hours using a preheated cabinet drier, set to 60 ℃

at a stretch for 8h. The rate of drying was carefully monitored, and the samples were pulverized and sieved through 40 mesh (0.4mm) and stored in airtight containers in the refrigerator at 4±2℃ for use. The images of underexploited seaweeds chosen for the study before and after processing were illustrated in Plate 3.1.

Seaweeds	Category	Before processing	After processing
Ulva lactuca	Chlorophyceae (green algae)		
Ulva reticulata			
Gracilaria edulis	Rhodophyceae (red algae)		
Sargassum polycystum	Phaeophyceae or Heterokontophyceae (brown algae)		

Plate 3.1. Underexploited seaweeds before and after processing.

Chemicals and Standards

All the chemicals, solvents, and acids used for the study were of the highest analytical grade and were procured from Sisco Research Laboratories Pvt. Ltd. and SD Fine Chem Pvt. Ltd., Mumbai, India, and Sigma-Aldrich, USA. All other chemicals used were of analytical grade obtained through commercial sources either from E. Merck or SRL India Limited. Double distilled water was prepared with a glass distillation apparatus and the same was used.

i. Macronutrient Profile.

The selected seaweeds were washed thoroughly in seawater and then in tap water. The seaweeds were again washed in distilled water, the remaining water was drained, and the fresh

seaweeds were dried in a cabinet drier at ~70℃, pulverized and sieved using 40 mesh. It is further used for nutrient and analysis. All marine algae are rich in nutrients and novel components when compared to higher plants (Fleurence, 1993). Quantitative estimation of proximate nutrients including moisture content, ash content, total carbohydrates, protein, fat, and crude fibre of *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis,* and *Sargassum polycystum* was analyzed in triplicates using standard estimation procedures given by National Institute of Nutrition (NIN, 2003). Moisture content was analyzed with Shimadzu Electronic Moisture Analyzer (MOC-120H). The Ash content of the charred samples was estimated using a muffle furnace. Total Carbohydrates were assessed with the Anthrone method wherein, protein was estimated using the Lowry method. Studies reported that Lowry and Lowry and biuret techniques of protein measurement are adequate for quantifying samples with low protein percentages, they are highly sensitive up to specified limits (Arunima *et al*; 2022). The fat content of the seaweeds was estimated by the Soxhlet method using Socs plus analyzer in petroleum ether (60°C - 80°C). The total, soluble, and insoluble dietary fibre content of the seaweed was estimated using the standard procedure given by the AOAC, 2011. The protocols are comprehended in Appendix III.

Control: Blanks in duplicate were also run along with samples to negate the effects of reagents on residue formation.

The nutrient content of the seaweeds was analyzed to understand the safety of the seaweeds to be used as raw or in a semi-processed form in the formulation of seaweed value-added food products.

ii. Micronutrient Profile.

The seaweeds contain a wealth of mineral elements. The micronutrients namely iron, phosphorus, calcium, zinc, and Vitamin-C, selenium, β-carotene, were analysed by standard AOAC methods (Raghuramulu *et al.,* 2003). The dried and ground seaweed samples were placed in a crucible overnight in an electric muffle furnace maintained at a temperature between 400 ºC and 420 ºC because the loss of zinc might occur at >450°C and potassium, loss might occur if the temperature is too high at >480ºC. Ashing will destroy all the organic materials present in the samples. The crucible containing pure ash was kept in desiccators. Then, the ash was digested with the triple acid mixture of nitric acid: sulphuric acid: and perchloric acid (11:6:3), and a clear solution was obtained when dissolved in HCl. This

solution was made up to 25 mL with water. The yield of the ash of each sample was determined by an Atomic Absorption Spectrophotometer (AAS). The standard protocols are presented in the Appendix III.

iii. Heavy Metal Profile.

Heavy metal toxicity was analysed with a selected method using an Atomic Absorption Spectrophotometer (AAS) of model Thermo Scientific ICE 3000 Series equipped with SOLAAR software and graphite tube atomizer. The sample preparation technique suggested by Mohammed *et al.,* (2017) was adopted with slight modifications. The dried seaweed samples of 0.5 g were predigested with 5N Nitric acid for 24 hours at room temperature. Predigested samples were digested at 100˚C until they dissolved completely and made up to 50 mL with triple distilled water. The obtained clear solutions were used for the analysis of heavy metals including mercury, cadmium, lead, chromium as well and arsenic, as reflected in the Appendix III. As per the mineral composition analysis, the edible seaweed with greater iron content (*Ulva lactuca*) is further chosen to develop the food product.

PHASE II

3.2 Development of the Seaweed-incorporated Probiotic Beverage.

The current study was executed at the Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore.

3.2.1 Procurement and processing of raw materials.

The preparation of the beverage consisted of ingredients like orange juice (40%), whey (55%), *Ulva lactuca* (variable) and palm jaggery powder (3%), and salt. Whey was extracted from homemade curd prepared using cow milk collected from a local milk vendor in Coimbatore. *U. lactuca* seaweed was harvested from the coastal area of the Gulf of Mannar and Ramanathapuram district, Tamil Nadu. Other ingredients were obtained from the local supermarket in the area. The dried *U. lactuca* powder was boiled for 10 minutes, and the concoction was further cooled to room temperature. Orange juice was extracted using a manual citrus juicer by Rlanos® and the beverage was prepared by adding whey, orange juice, and palm jaggery powder to the pre-processed seaweed concoction.

Oranges (Nagpur orange - a variety of mandarin orange *Citrus reticulata* were chosen for the food product development as a natural source of Vitamin C and for enhanced sensory

appeal. Ascorbic acid is a well-known dietary factor that improves iron bioavailability. The addition of ascorbic acid was observed to counteract the inhibitory effect of phytates, which are a common iron inhibitor in plants. Some intervention studies administering products rich in vitamin C in conjunction with iron sources showed improved iron status. Studies revealed that when compared to consuming a 100 mg iron dose in the morning with coffee or breakfast, consuming it with orange juice alone results in a ~ 4-fold increase in iron absorption, and provides ~20 more mg of absorbed iron per dose (Hanna *et al.,* 2023).

Whey, instead of milk was used to avoid the active hindrance of calcium for iron bioavailability. Whey, a by-product of curd production, has been recognized for its probiotic content, particularly strains of *Lactobacillus* (Kechagia *et al.,* 2017). Recent studies indicate that whey contains beneficial microbes such as *Lactobacillus casei* and *Lactobacillus acidophilus*, which have potential health benefits (Shi *et al.,* 2019).

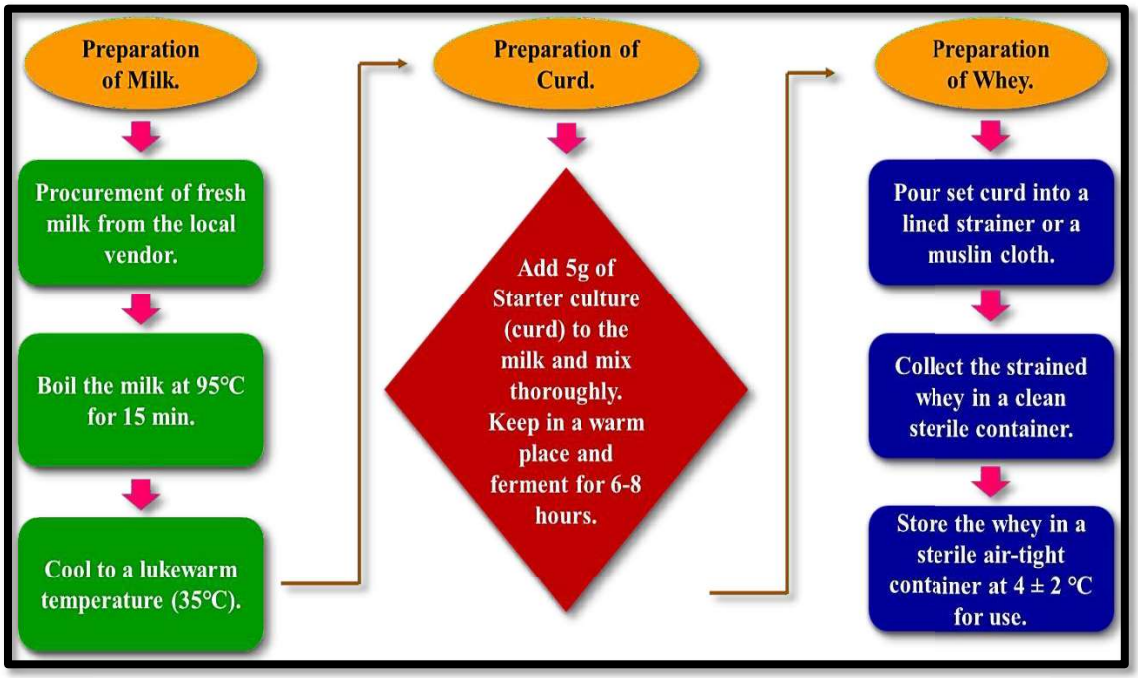

Fig. 3.3. Preparation of Whey for the Development of the Beverage.

Lactobacillus reuteri has been studied extensively for its role in enhancing iron bioavailability. Szulińska *et al.,* (2018) demonstrated that *L. reuteri* improves iron absorption in humans by producing organic acids that lower intestinal pH, facilitating iron solubility. Similarly, a study by Freitas *et al.,* (2019) found that *L. reuteri* enhances the expression of iron transport proteins in the gut, thus improving iron uptake. Hugenholtz *et al.,* (2016) reported

that *L. reuteri* can bind iron ions, forming complexes that are more readily absorbed by the intestinal mucosa. Figure 3.3. depicts the preparation of whey for the development of probiotic beverage in the current study.

Karupatti or Palm jaggery which is obtained from the juice or sap of Palmyra palm (*Borassus flabellifer L.)* aided as a source of Iron along with the seaweed in the beverage. Palm jaggery, a traditional non-centrifugal cane sugar, has gained attention for its potential role in enhancing iron bioavailability. Gopalakrishnan *et al.,* (2017) conducted an *in vitro* study demonstrating that palm jaggery significantly increased the bioavailability of iron compared to refined sugar, suggesting its potential utility in addressing iron deficiency. Similarly, Jain *et al.,* (2018) reported that the consumption of palm jaggery resulted in higher serum iron levels in anaemic rats, supporting its use as a dietary supplement for improving iron status. A comprehensive review by Das *et al.,* (2023) synthesized findings from multiple studies, concluding that palm jaggery is a viable dietary intervention for combating Iron Deficiency Anaemia due to its superior iron bioavailability.

U. lactuca, a green seaweed was collected from Ramanathapuram District's sea shore, including the Gulf of Mannar and Palk Bay. The sea coasts of the Gulf of Mannar and Palk Bay in Ramanathapuram District have abundant marine algal growth. *Ulva lactuca* could be exploited for its multifunctional properties in the form of food, energy, medicine, and as biotechnological tools. *Ulva lactuca,* has garnered considerable attention for its potential in enhancing iron bioavailability, a critical factor in addressing iron deficiency. Recent studies have highlighted that the polysaccharides in *Ulva lactuca* can act as natural chelating agents, improving iron solubility and uptake (Li *et al.,* 2017). Studies indicate that seaweed concoction significantly improves non-heme iron absorption by breaking down complex polyphenols and bioactive compounds into absorbable forms, thus enhancing the nutrient bioavailability of beverages. (Ganesan *et al.,* 2017; Hardikar *et al.,* 2019). Additionally, *Ulva lactuca's* bioactive compounds facilitate iron uptake in Caco-2 cells (Kim *et al.,* 2020).

3.2.2 Formulation and standardization of the seaweed-incorporated probiotic beverage.

Clean and sterile apparatus and instruments were used for the food product development in the food processing laboratory.

The standard sample did not contain *U. lactuca* whereas the subsequent variations contained different concentrations of the seaweed. The formulated beverage was standardized by trials to obtain optimum sensory appeal and iron bioavailability. Variation 1 (V_1) contained 2% dry wt. *U. lactuca*, Variations 2 (V_2) and 3 (V_3) subsequently contained 3% and 4% of *U. lactuca*. Plate 3.4 shows the composition of the variants of the developed probiotic beverage. Table 3.1. shows the composition of the various ingredients used in the preparation of the beverage. The process flow chart of preparation of *U. lactuca*-based probiotic beverage is portrayed in Figure. 3.4.

Table 3.1. Percentage composition of the ingredients used in beverage preparation.

Ingredients	Percentage Composition			
	Standard	**V_1**	**V_2**	**V_3**
Orange juice	40.0	40.0	40.0	40.0
Whey	55.0	55.0	55.0	55.0
Palm jaggery	3.0	3.0	3.0	3.0
***U. lactuca* extract**	Nil	10.0	15.0	20.0

The Recommended Dietary Allowance (RDA) of iron for Indians varies based on age, sex, and physiological status, with adult men requiring 17 mg/day and women 21 mg/day (ICMR, 2020). Research indicates that beverages enhancing iron bioavailability should provide at least 10-15% of the RDA per serving to be effective (Chaudhary *et al.,* 2017). Gupta *et al.,* (2019) demonstrated that fortifying beverages with ascorbic acid significantly improves iron absorption. Additionally, studies by Singh and Sharma (2021) revealed that iron-fortified beverages can bridge dietary gaps in populations with high anemia prevalence. Shukla and Mishra (2018) found that polyphenol-rich drinks inhibit iron absorption, suggesting the need for careful formulation. Furthermore, an investigation by Desai *et al.,* (2020) emphasized the importance of considering both iron type and beverage matrix to optimize bioavailability. Plate 3.2. shows the various compositions of the developed probiotic beverage.

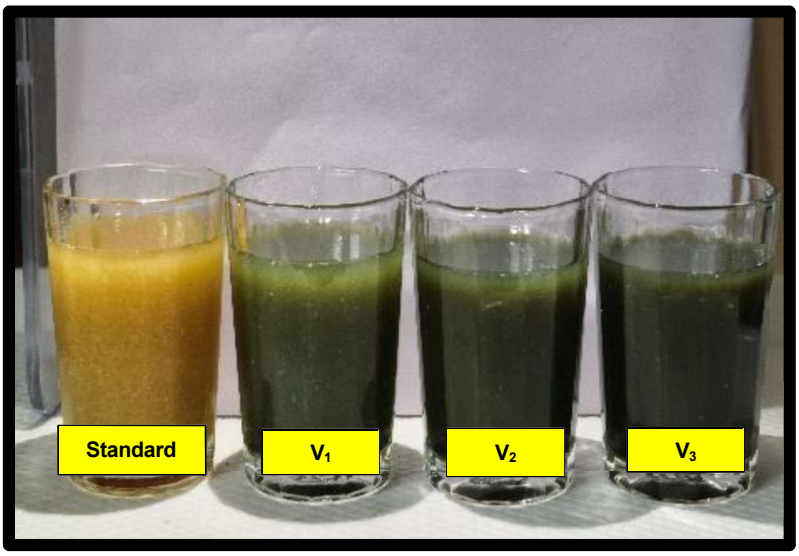

Plate 3.2. Various compositions of the developed probiotic beverage.

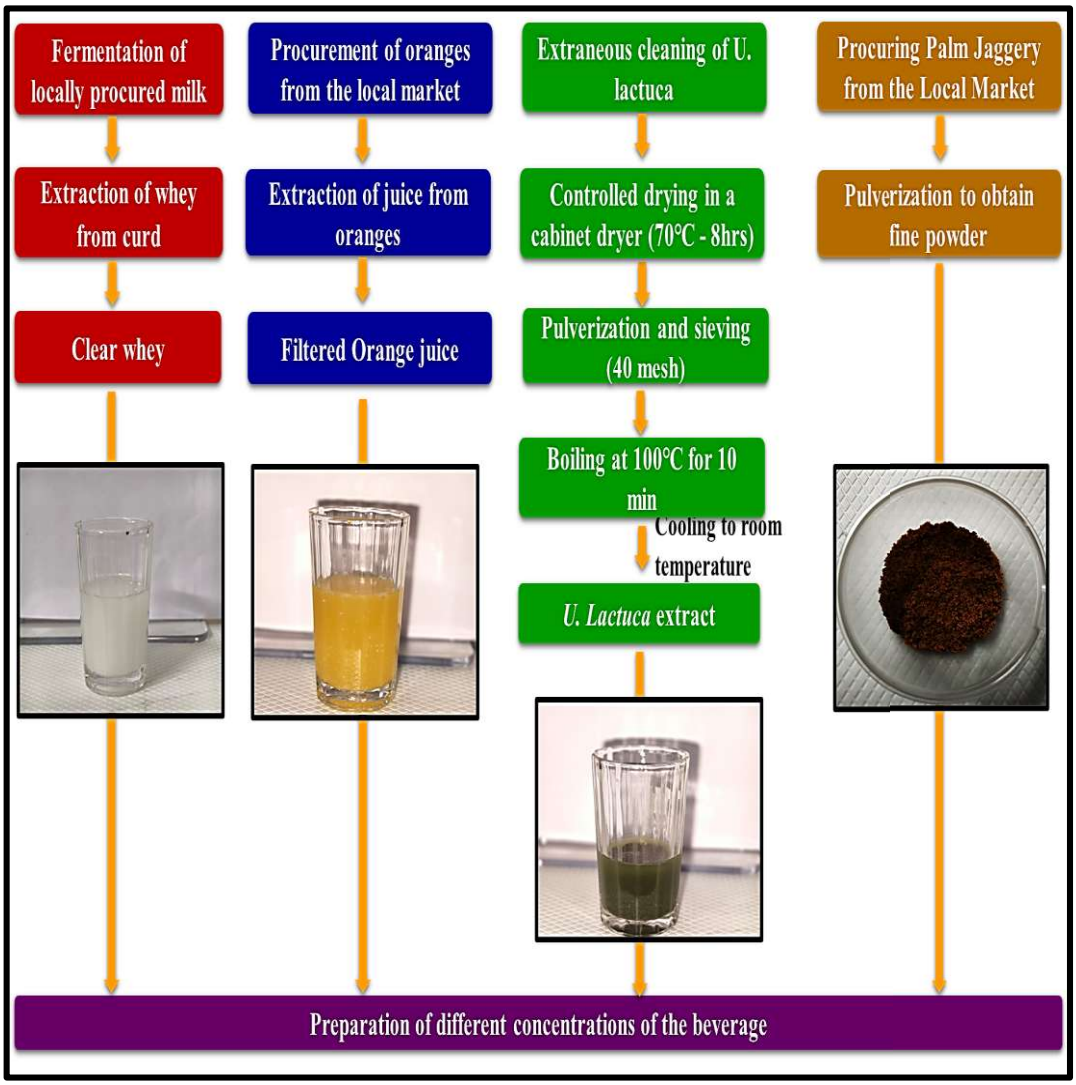

Fig 3.4. Preparation Flow Chart of Probiotic Beverage containing *U. lactuca*.

3.2.3 Sensory Evaluation of the Developed Beverage using a 9-point Hedonic Scale.

The evaluation of food in sensory evaluation is done through all the senses as the individual interacts with the food being examined. The usual examination involves a judgment on both texture and colour. The smell sense is used to assess the aroma of the meal and influences how flavour is perceived in general. The ability to distinguish between sour, sweet, salty, and bitter flavour components depend heavily on one's sense of taste (Srilakshmi, 2010).

The organoleptic study was carried out to determine the organoleptic properties of the developed product. The sensory examination was conducted with a total of 34 qualified panel members. The panelists were shown the newly prepared products. To eliminate prejudice in taste, the panelists were given a glass of water. They were asked not to consume any other highly flavoured meal for at least one hour before the test to avoid taste interaction that might impact the evaluation choice. The variations were labelled as V_1, V_2, V_3, and standard. The indicated quality parameters such as colour, taste, appearance, flavour, and overall acceptability were assessed using a 9-point hedonic scale. The nine-point hedonic scale ranges from 1= Dislike extreme and 9 = Like extreme (Lawless *et al.,* 2013). It is also known as the grade or taste scale. The components used, the proportions, and the cause of variation were not given to the panelists to reduce any taste expectations. All panelists were given separate sheets with instructions for evaluating the product. The variation with the highest overall score was considered the best-accepted variation and it was chosen for further analysis.

PHASE III

3.3 Determination of Physical Characteristics, Nutrient, and Nutraceutical Potentials of the Developed Beverage.

The beverage was further assessed for the proximate composition of the nutrients, and probiotic potential as per the guidelines prescribed by ICMR, 2016.

3.3.1 Assessment of Physical Characteristics of the Developed Beverage.

The physical properties of the probiotic beverage are assessed using Quality parameters like pH, titratable acidity, and viscosity. These factors determine the stability of the bioactive compounds present in the beverage. The pH of the beverage was measured using Lab Care Export Digital PH Meter LB-901. Viscometer-Brookfield Model RVDI, (USA) was used to quantify viscosity. The percentage of lactic acid was used to determine titratable

acidity, which was executed by titrating with 0.1 NaOH, with phenolphthalein as an indicator. Brix was measured using a Digital refractometer (Rudolph, USA).

3.3.2 Assessment of Nutritional and Nutraceutical Profile of the Developed Beverage.

i. Estimation of Proximate Nutritional Composition of the Developed Beverage.

Chemicals and standards

Reagents or chemicals namely α-amylase, amyloglucosidase, glucose oxidase peroxidase reagent, sodium hydroxide, potassium hydroxide, sodium carbonate, phytic acid, α-tocopherol, Folin-Ciocâlteu reagent, total dietary fibre kit (TDA-100A, Lot SLBP2362V) was procured from Sigma-Aldrich, USA. All other chemicals, solvents, and acids were of the highest analytical grade and were procured from Sisco Research Laboratories Pvt. Ltd. and SD Fine Chem Pvt. Ltd., Mumbai, India.

Nutritional parameters were estimated as per the standard protocol followed while analyzing seaweeds, as stated earlier.

Determination of the dietary fibre

Soluble components of dietary fibre, like pectin and gums, form complexes with iron, potentially increasing its solubility and absorption in the gut. In contrast, insoluble fibres, such as cellulose and hemicellulose, can bind to iron and reduce its availability for absorption. Therefore, understanding the fibre composition in foods is crucial for optimizing iron intake and ensuring adequate nutritional balance,

To determine dietary fibre in the beverage, the sample is first enzymatically digested to break down soluble components. Insoluble fibre is filtered and washed to remove non-fibre components. The residue undergoes precipitation of proteins and non-fibre carbohydrates, followed by drying and incineration to leave behind the fibre content. Dietary fibre is calculated as the difference between the weight of the total residue and the protein/nitrogen-free extract. This method ensures accurate estimation of dietary fibre content in beverages, crucial for nutritional labelling and health assessments. Dietary fibre was determined using the enzymatic and gravimetric - AOAC 985.29 method (AOAC, 1997), and is elaborated in the Appendix III.

Determination of ferritin iron content

Ferritin iron was determined according to the method explained by Zielinska-Dawidziaka *et al.,* (2014) one mL of the beverage was extracted with the 20 mL of 6M HCL for 30 min at 80 ºC. Extracted inorganic iron, not chelated, and not introduced into organic compounds, was determined after thiocyanate reaction spectrophotometrically (480nm) at first and after oxidation reaction as a sum of Fe^{3+} and $Fe^{2+.}$

ii. Assessment of Qualitative and Quantitative Phytochemical Profile.

Marine algae are among the richest sources of known and novel bioactive compounds with valuable antioxidant potentials and application as therapeutic agents (Strayo *et al.,* 2005). The Total Phenolics (TP) were determined according to the Folin-Ciocâlteu method adapted for microplate assay (Zhang *et al.,* 2006). Total flavonoid (TF) content was estimated in the same extract used to determine TP and quantified using the method given by (Kim *et al.,* 2003) for the spectrophotometry method. Tests for quantitative estimation of phytochemicals like oxalates and alkaloids were carried out using standard procedures elucidated in the Appendix III. Alkaloids by Hager‘s test and phytosterol by Salkowski‘s test were carried out with the standard procedure of Tiwari *et al.,* (2011), for qualitative estimation.

Calculation of the phytochemical quality index

To quantitatively evaluate the overall phytochemical quality of the beverage, an Overall Phytochemical Composite Index (OPCI) was devised based on the relative concentrations of four phytochemicals – total polyphenols, total flavonoids, total oxalates, alkaloids. Equal weights were assigned to all the phytochemical assays. First, individual phytochemical indices were computed separately by assigning a score of 100 to the highest value for each method and subsequently calculating the score for all the other samples as a percentage of the highest score e.g., Total phenolics index = Total phenolic concentration/ highest concentration of TP x 100. The OPCI was then calculated as the average of the five phytochemical indices.

Micronutrient and Antinutrient Quality Score (MQS).

The percent nutrient contribution (NC) (Equation) to the meal was then calculated based on the reference daily values (RDV) for protein, dietary fibre and Na, and reference daily intake (RDI) for the minerals (Ca, Mg, K, Fe, Zn and Se) and vitamins (vitamin C, E and

A) (FDA, 2016). In the case of the antinutrient, oxalate, a maximum recommended value (MRV) of 200 mg oxalate/ day was considered (Coe, 2019). Beta-carotene content were expressed as retinol activity equivalents (RAE) (Appendix III), where 12 µg β-carotene = 1 µg retinol = 1 RAE. The equations used for computing EDI and NC are given below:

The NQS was computed based on the sum of percentages of nutrient contribution for 11 nutrients to encourage (Ca, Mg, K, Fe, Zn, protein, dietary fibre, ascorbic acid, vitamin A and vitamin E) minus the sum of percentages of the nutrient contribution of sodium and maximum recommended value (MRV) of OxA, the nutrients to limit (Eq. 11). The percent nutrient contribution of the nutrients was capped at 100 % so that contribution of a single nutrient would not result in disproportionately higher nutrient score. The NQS was computed using the following algorithm:

$$\boldsymbol{NQS\ 11.2} = \Sigma(\%\ \boldsymbol{NC})\boldsymbol{1-11} - \Sigma(\boldsymbol{EDIi\ MRV\ i})\boldsymbol{1-2x\ 100} \quad \text{(Eq.)}$$

Where,

EDIi – Estimated daily intake

% NC – Percent nutrient contribution for the 11 nutrients to encourage

MRV – Maximum recommended for OxA

iii. Determination of *In vitro* Antioxidant Potential of the Developed Beverage.

The various antioxidant assays described below were determined in the same sample extract used for the total phenolics assay.

a. DPPH Radical Scavenging Assay

The antioxidant activity of the beverage was measured based on the scavenging activity of the stable 1, 1- diphenyl 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the procedure as described by Brand-Williams *et al.,* (1995) with slight modifications. ―DPPH is a stable free radical with deep violet colour which turns yellow once scavenged by an antioxidant compound. The DPPH assay uses this characteristic property to show free radical scavenging activity. The degree of discolouration indicates the scavenging potential of the antioxidant compound present‖ (Brand-Williams *et al.,* 1995). Aqueous extracts of the beverage were used for assessing the radical scavenging activity using a standard procedure, described in the Appendix III.

b. Ferric reducing antioxidant potential (FRAP)

The FRAP assay determines the capacity of antioxidant compounds present in the sample extract to reduce ferric-TPTZ complex to the ferrous form at low pH. It was determined according to the microplate method described by Lister *et al.,* (2020).

Sample estimation:

To 7.5 µl of the sample extract, 142.5 µL of FRAP reagent (300 mM acetate buffer at pH 3.6; 10 mM 2,4,6-tripyridyl-s-triazine; 20 mM ferric chloride in the ratio of 10:1:1 respectively) was added. It was incubated for 6 minutes at room temperature and absorbance of the reaction mixture was measured at 593 nm. Ferrous sulphate was used as the standard (100 – 1000 µmol/L). Ascorbic acid was used as the positive control. Results were expressed as Ferrous Equivalent (µmol FE/g FW).

iv. Estimation of Bio-active Compounds Profile using GC-MS/MS.

For the derivatization, 100 µL of 20,000 ppm pyridine was added to 5 mg of each lyophilized sample and incubated at 30 °C for 90 min. Next, 100 µL of N, O-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane solution and 20 µL of internal standard (1000 ppm fluoranthene) were added to the sample vial and incubated at 60°C for 30 min. The samples were analyzed through GC-MS. GC chromatographic separations were achieved on a Thermo Scientific TRACE™ 1310 Gas Chromatograph with a single quadrupole mass spectrometer. The GC was equipped with a capillary column (Agilent, Palo Alto, CA, USA; DB-5MS 60 m × 0.25 mm × 0.25 µm) and run in full-scan mode (scan range 40–700 m/z with detector voltage 2160 V). The gas carrier was helium, with a flow rate of 1.5 mL/min. The transfer line was settled at 310 °C and 1 µL of the sample was injected. The oven temperature was fixed at 50 °C for 2 min, increased to 180 °C (rate 5 °C/min), held for 5 min, increased to 325 °C (rate 5 °C/min), and held for 10 min. The ion source was settled at 270 °C and the solvent delay was 4.5 min. Mass spectra were recorded in electronic impact mode at 70 eV, scanning within the 35–650 m/z range for the selection of appropriate electronic impact mass fragments for each analyte, with a scan rate of 6 scans/s. The standard solutions (pyroglutamic acid, γ-aminobutyric acid, and gluconic acid) were obtained from Sigma Aldrich (St. Louis, MO, USA). For MS/MS analysis the data were acquired using the extended dynamic range mode (2 GHz) and an automated full scan mass covered the range of m/z 50 to 1500.

Data mining and analysis

Agilent Personal Compound Database and Library (PCDL) was utilized to generate a database with the phenolic compounds obtained from ChemSpider (http://www.chemspider.com/), Phenol-Explorer (http://phenol-explorer.eu/), PubChem (https://pubchem.ncbi.nlm.nih.gov/) and FoodB (https://foodb.ca/). Bioactive compounds with scores greater than seventy were documented and considered for further study. The experimental retention time (RT), of the identified bioactive compounds was compared with literature values and theoretical values.

***a. In silico* ADME Profile of Abundant Bioactive Compounds.**

The significance of *in silico* ADME (Absorption, Distribution, Metabolism, and Excretion) profiling for abundant bioactive compounds lies in its ability to predict pharmacokinetic behaviours efficiently and cost-effectively. Recent studies have underscored the utility of these computational models in drug discovery. For instance, *in silico* ADME analysis has been instrumental in identifying potential drug candidates by predicting their solubility, permeability, and metabolic stability (Di *et al.,* 2017). Additionally, Singh *et al.,* (2018) highlighted that such models could accurately forecast human intestinal absorption, enhancing early-stage drug development decisions. Jain and Sinha (2019) emphasized the role of *in silico* techniques in optimizing lead compounds by predicting their interactions with biological targets. Moreover, Kumar *et al.,* (2020) demonstrated the efficacy of these models in predicting hepatic clearance and potential drug-drug interactions. Notably, Zhang *et al.,* (2021) found that in-silico ADME profiling could reduce the reliance on animal testing, aligning with ethical considerations. Lastly, a study by Lee *et al.,* (2022) corroborated the predictive accuracy of these models in assessing the pharmacokinetic properties of natural products, thus facilitating the exploration of novel therapeutic agents.

Ligand generation.

The 2D structures of the identified phyto-compounds (ligand molecules) from the beverage through GC - MS analysis were drawn in ACD – Chemsketch (ACD/ ChemSketch Freeware, Version11, 2006) and their SMILES notation was obtained. The SMILES notation was submitted to online SMILES converter and structure file generator and further converted to 3D PDB file format (Weininger 1988). The obtained 3D PDB files and SMILE notations of ligands were utilized for further study (Thirumalaisamy *et al.,* 2018).

Drug likeness and pharmacokinetic properties.

The drug-likeness nature of the abundant phyto-compounds was analyzed using SWISS ADME online server and examined based on the violations of rules such as Lipinski, Ghose, Veber, Egan, and Muegge. The phyto-compounds pervading zero violations were subjected to the next level of *in silico* virtual screening. To predict the various pharmacokinetic properties associated with such as Adsorption, Distribution, Metabolism and Excretion (ADME), the phyto-compounds were evaluated through web-based applications namely Swiss ADME and QikProp module of Schrodinger Maestro 9.3 version.

PHASE IV

3.4 Assessment of *in vitro* probiotic potential, safety assessment and antimicrobial activity of the Lactic Acid Bacteria in the beverage.

3.4.1 Isolation and identification of probiotic bacteria in the beverage.

Isolation of the Probiotic Strain.

Lactic acid bacteria were isolated from the developed probiotic beverage by standard plate count method using de Man, Rogosa and Sharpe agar (MRS) medium. From each sample, a 1:10 dilution was subsequently made using sterile normal saline water (0.85%) followed by making a 10-fold serial dilution. The 0.1 mL from each dilution was then sub-cultured aseptically into MRS agar (Guessas and Kihal, 2004) using the streak plate technique. All plates were then incubated at 37 °C for 24 to 48 h in anaerobic conditions to provide an optimal environment for growing lactic acid bacteria. Colonies that differ in morphology, pigmentation, shape and size were sub-cultured in MRS broth. Initially, all the isolates were examined for Gram staining. Only the Gram-positive isolates were then purified by streak plate method on medium. The culture was kept in MRS agar slant and stored at 4 °C for further use (Hawaz *et al.,* 2014).

Identification of the Isolated Probiotic Strain.

The identification and further characterization of *Lactobacilli* isolates grown on MRS agar was done mainly with the help of the following tests: microscopic examination (Gram staining), catalase test, growth at different temperatures 10 + 1 ºC and 42 + 1 ºC), growth under aerobic and anaerobic conditions, growth at different NaCl concentration, fermentation of different carbohydrates, etc.

Microscopic Examination

The purity morphological identification of the isolates as Lactobacilli was confirmed microscopically by performing Gram staining, for which a single colony of each isolate was picked up and stained as per the standard protocol and viewed under oil immersion for similar types of cells.

Micrometry

Each isolate after Gram staining was subjected to microscopic measurements employing ocular and stage micrometer. To determine the size of *Lactobacilli* isolates, prepared slides were observed under the oil-immersion objective and the number of ocular divisions occupied by each bacillus was recorded and interpreted accordingly.

Physiological Characterization of Isolates

After confirming the purity of the culture, each isolate was further assessed for growth at two different temperatures. Growth of isolates at (10 °C and 42 °C): The isolates were tested for their ability to grow in MRS broth at 10 + 1 °C for 7 days and 42 °C by incubating for 24–48 h. For this, 10 mL of MRS broth tubes were inoculated at 1% of *Lactobacilli* cultures. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at 10 °C and 42 °C and results were noted as positive or negative.

Oxygen requirement of the isolates: All the isolates were inoculated in MRS broth and were kept differently under oxygenated condition; in dessicator with burned candle (for micro-aerophilic condition) and in anaerobic jar with gas pack at 37 °C for 24–48 h to determine the impact of oxygen on the growth of the *Lactobacilli* isolates and results were noted as positive or negative.

Effect of NaCl Concentrations on Growth of Isolates

The isolates were inoculated in MRS broth having different NaCl concentration (2.0%, 4.0% and 6.5%) and incubated at 37 °C for 24–48 h. The culture tubes were observed for the presence or absence of growth.

Biochemical Characterization of Isolates

Endospore test, motility test, Indole test, Vogues-Proskauer, Methyl-red, catalase and Citrate Utilisation tests were done to characterize the presence of the isolated *Lactobacillus*

strain from the developed Probiotic beverage. The protocols of the tests were elaborated in Appendix III.

Morphological Identification of the Isolated Probiotic Strain.

Reagents and Standards

All the reagents used for DNA extraction, purification and PCR amplification were of molecular biology grade and purchased from Sigma-Aldrich (Bangalore, India). Oligos were synthesizes in BioServe Biotechnologies Pvt. Ltd., Hyderabad (India).

The probiotic beverage containing *U. lactuca* was aseptically prepared and the sample was collected in a container that was pre-sterilized in the laboratory. According to the conventional procedure gleaned from Liu *et al*., 2019, serial dilution of the sample was done at the power of 10^{-3} times, and 0.1 mL of the strain isolate was pour-plated into MRS agar and incubated at 37 ℃ for 24h under anaerobiosis. After acquiring distinct colonies of organisms, pure cultures were acquired by sub-cultivation onto MRS agar plates. (Zhou *et al*., 2021).

Colony morphology, characteristics of the isolated culture, and microscopic observations were carried out as a part of the preliminary identification protocol. Biochemical screening assays were conducted on the isolates from the differential media and the results were systematically obtained. These include testing for urease, the Vogues-Proskauer test, the H_2S generation test, the indole production, citrate utilization, and arginine hydrolysis testing. According to Kunchala *et al*., 2017, Thakur *et al*., 2017, and Obasi *et al*., 2019, evaluations for starch hydrolysis, carbohydrate fermentation, oxidase, and Voges-Proskauer were also conducted.

Phylogenic Tree Analysis.

BioEdit Sequence Alignment Editor Version 5.0.9 was used to assemble and edit forward and reverse sequences. BLAST was used to search for homology in the Genebank DNA database to estimate sequence similarity. Finally, the isolate was identified using the sequence. In order to construct a phylogenetic tree, the neighbor joining method (Saitou and Nei, 1987) was used with the program PHY-LIP (version 3.64). The optimal tree is shown, with a branch length sum of 0.19972900. The percentage of replicate trees in which the interrelated taxa clustered together in the bootstrap test (1000 replicates). (Felsentein, 1985) is

displayed next to the branches of the tree. The phylogenetic tree was drawn with the same units of lengths of branches with evolutionary distances in scale. The Evolutionary analyses were conducted in MEGA 7 and computed with a number of base substitutions and eliminating the gaps and missing data.

In lieu of the process elucidated by Angelescu *et al.,* 2019, extraction of the DNA isolate was carried out and its characteristics were determined. As per the protocol, the ‗universal primers' 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′TACGGYTACCTTGTTACGACTT-3′), were utilized and bacterial 16S ribosomal RNA-based polymerase chain reaction was meticulously executed. (Plessas *et al.,* 2017). A comparative study approach was used to analyze the 16S rRNA gene sequence homologies. A BLAST search was used to match the obtained sequences to those in the NCBI repository. The gene sequences were examined, and a tree portraying the evolutionary relationship was structured using MEGA 6.0 software (Mulaw *et al.,* 2019).

3.4.2 Assessment of *in vitro* Probiotic Potential.

Resistance to gastric acidity of food mixes at different pH values at different exposure times was done by standard protocol given by Awan and Rahman (2005). The procedure outlined by Nakkarach *et al*., 2018 was followed for conducting the bile tolerance test. For the pancreatin tolerance test, the cell viability of the test and control specimen in MRS agar plates was also calculated as per the protocol suggested by Khagwal *et al.,* 2019 and Gebre *et al.,* 2023. The Surface-Hydrophobicity Index was calculated as per the protocol suggested by (Reuben Roy *et al.,*2019). NaCl tolerance and Auto Aggregation Assay were carried out as per the methods given by Yépez *et al.,* 2019; de Oliveira Coelho *et al.,* 2019 and Byakika *et al.,* 2020 respectively. The procedures of the tests were elaborately discussed in the Appendix III.

Characterization of Carbohydrate Fermentation.

A specific sugar was added to the basal carbohydrate media and the obtained isolates were further evaluated for their ability to ferment other sugars like glucose, fructose, lactose, sucrose, xylose, maltose, galactose, and ribose. A change in coloration showed the formation of acid, and the inverted Durham tubes' gas collecting revealed the production of gas (Kowalska *et al.,* 2020).

3.4.3 Safety Assessment and antimicrobial activity, storage, and shelf-life studies.

The safety assessment and antimicrobial activity, along with storage and shelf-life studies, are critical aspects in the evaluation of probiotic beverages. The safety of probiotic beverages is paramount, as highlighted by Patrignani *et al.,* (2016), who underscored the need for thorough toxicological evaluations to prevent adverse health effects. Similarly, Yan *et al.,* (2020) emphasized that probiotic strains should be non-pathogenic and free from harmful antibiotic-resistance genes. Antimicrobial activity of probiotics is another significant aspect. According to Zommiti *et al.,* (2018), certain probiotic strains exhibit strong antimicrobial properties against pathogens, which enhances their therapeutic potential. This finding is supported by Wang *et al.,* (2019), who demonstrated the effectiveness of *Lactobacillus* strains in inhibiting foodborne pathogens.

Storage and shelf-life studies are essential for maintaining the efficacy of probiotic beverages over time. Nag *et al.,* (2020) reported that the stability of probiotics during storage is influenced by factors such as temperature and packaging materials. Furthermore, the work of Succi *et al.,* (2017) revealed that the sensory properties of probiotic beverages are maintained over prolonged storage, which is crucial for consumer acceptance. Studies by Kamil *et al.,* (2019) indicated that regular monitoring and quality control during the storage period are essential to ensure the safety and functionality of probiotic beverages. Additionally, Suo *et al.,* (2019) pointed out that proper storage conditions could prevent the degradation of bioactive compounds, thereby maintaining the health benefits of the beverage. The comprehensive evaluation of the safety, antimicrobial activity, and storage stability of probiotic beverages is vital for their successful commercialization and health efficacy. These studies provide a scientific basis for the development of safe, effective, and stable probiotic products.

i. Haemolytic Activity

Determination of haemolytic activity is carried out as suggested by Romero-Luna *et al.,* 2020 Mousanejadi *et al.,* (2023). Antibiotic Susceptibility of *L. Reuteri* on Common Enteropathogens was based on the disc agar diffusion suggested by Cui *et al.,* (2018) with minimal alterations. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella typhi*, and *Escherichia coli* were the enteropathogens tested via well diffusion

method to study the growth inhibitory effects of *L. reuteri* against the fore-mentioned test pathogens. According to Zhang *et al., (*2020), the size of the growth-inhibiting zones was scaled and categorized as sensitive (18±2 mm), intermediately sensitive (10-18 mm), and resistant (>10 mm).

ii. Antibiotic Susceptibility.

The antibiotic susceptibility test (AST) for the isolate was conducted using the Kirby Bauer disc diffusion method with plates embedded with antibiotic discs (Himedia, India) viz.; Amoxicillin sulbactam- AMS 30/15 mcg, Penicillin – P 10mcg, Amoxiclav– AMC 10mcg, and Chloramphenicol – C 30 mcg, Gatifloxacin – GAT 5mcg, Moxifloxacin –MO 5mcg, Methicillin – MET 5mcg and Ceftazidime – Cz 30mcg. The plates were then incubated at 35° ± 2 °C for 48 h to assess the strain susceptibility to the selected antibiotics. The zone of inhibition was measured for each isolate, and the interpretation of the results was based on the diameter zone as per Performance Standards for Antimicrobial Disc Susceptibility Tests.

iii. Antagonistic activity of the Extracted Antibacterial Agents and Calculation of the Minimum Inhibition Concentration (MIC).

LAB isolates were screened for their antimicrobial activities against selected foodborne pathogens. In this investigation, *S.aureus, P.aeruginosa, B.cereus, S.typhi,* and *E.coli,* which are the most common enteropathogens affecting gut health, were tested by the LAB isolate. Well-diffusion method was adopted on Muller Hinton Agar (M187-500 G, HiMedia, New Dell, India), while the tested pathogens were incubated at 37 °C for 24 h. Following incubation, the plates were examined for the presence of a zone of inhibition surrounding the well area and the Minimum Inhibition Concentration was meticulously estimated.

3.4.4 Determination of Antimicrobial Activity, Storage and Shelf-life Study.

Antimicrobial activity was carried out by disc diffusion method (Daoud *et al.,* 2015). The microbial safety of the selected films was assessed using pH, titratable acidity, Total Viable Count (TVC), Total Bacterial Count and Total Fungal Count of the beverage with the standard method. IS 5402:2012/ISO 4833:2003, BAM, DGHS Manual 2005 (Beuchat *et al.,*1998) was used to number the colonies that were grown in a solid medium following

aerobic incubation around 35 °C and microorganisms were assessed after 2, 4, 6, 8 and10 days of refrigerated storage at 4±2 ℃, as detailed in the Appendix III.

PHASE V

3.5 Assessment of in-vitro bioavailability of iron from the selected seaweed and developed beverage using Caco-2 cell model.

3.5.1 *In vitro* stimulated Digestion.

In vitro digestion was performed according to the cell-free model described by Miller et al with modification described by Venkatasubramanian *et al.,* which was suitable for the 6-well plate. In brief, each sample was subjected to pepsin digestion (1.60 g of pepsin made up to 10 mL with 0.1 N HCI) by incubating in a shaking water bath for 2 h at 37 °C, 200 rpm. Two grams of pepsin digestate was used to perform intestinal digestion with 0.5 mL pancreatin-bile salt mixture (4 g of pancreatin and 25 g bile suspended in 0.1 M NaHCO3) was added and titrated against 1 M NaHCO3 till pH increased to 7.5.

i. Dialysis.

Dialysis was carried out in 6-well plate (Thermo Fisher) using 5 cm long dialysis tube (nominal MW cut-off 6000–8000 Da). The opened dialysis membrane was fixed to the base of glass inserts using latex elastic system and placed back in distilled water. Volume of 1 M NaHCO3 equivalent to that of titratable acidity was added to each well and made up to 2.5 mL with distilled water. The pepsin digest (2g) obtained from the above step was taken on the upper chamber of the insert and was placed on the well with the membrane just immersed in the 1 M NaHCO3.The plates were then incubated on a shaking water bath at 37 °C for 30 min. When the pH of the digest increased to 5, a pancreatin-bile salt mixture (0.5 mL) was added to the samples and the incubation was continued for 2h in shaking water bath at 37°C. At the end of this incubation period, the insert with the digest was removed. The dialysate was collected from the well and made up to 2.5 mL with distilled water and stored at -80 °C until further use. Ferrozine based colorimetric method was adopted for the estimation of iron content in the dialysate.

3.5.2 Cell Culture.

The Caco-2 (Human colorectal adenocarcinoma cell line) was purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in

Dulbecco's Modified Eagle Medium (DMEM) high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured every 2 days. Passage No-21 was used for the current study. Figure 3.5. demonstrates the schematics of a two-chamber system for assessment of iron bioavailability using Caco-2 cell model.

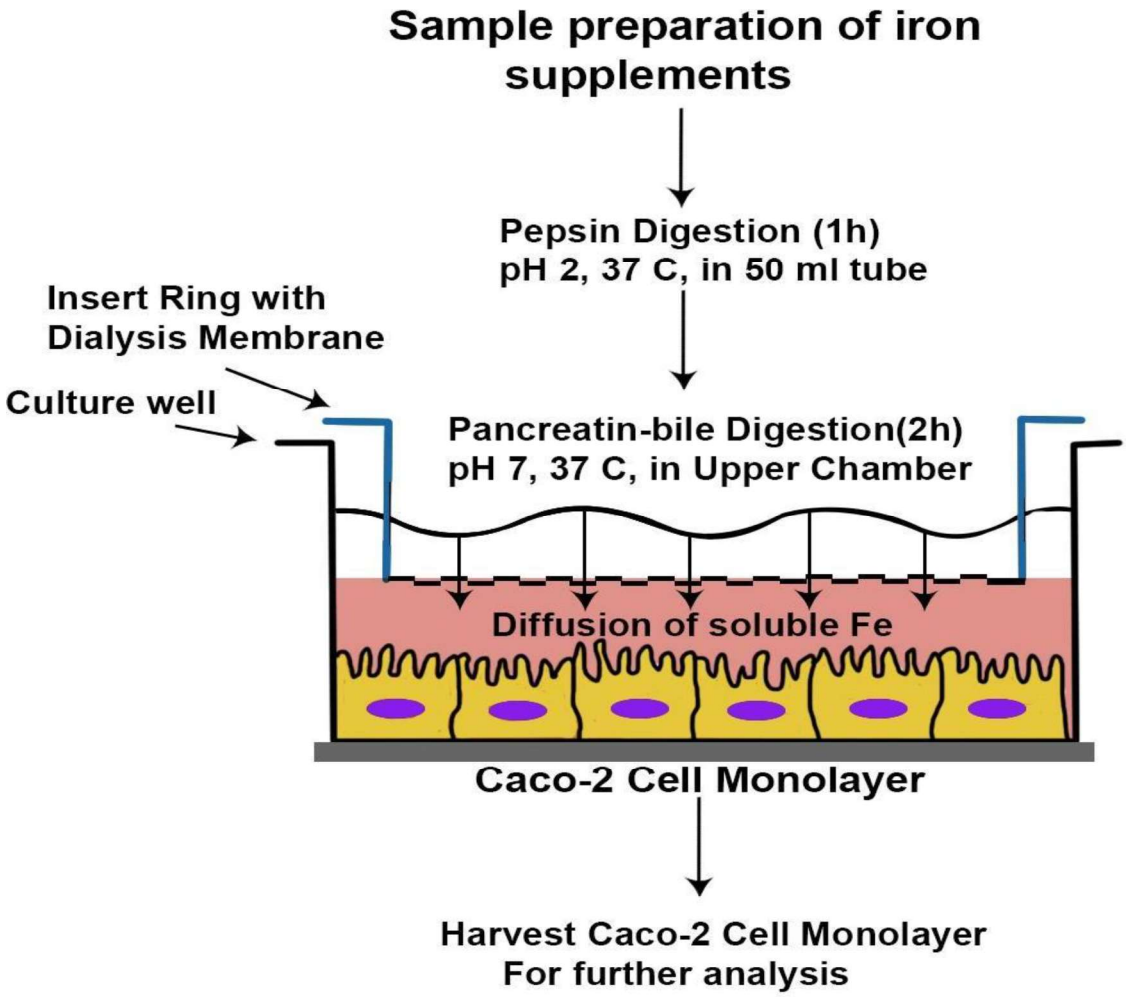

Fig 3.5. Schematics of a two-chamber system for bioavailability assessment.

3.5.3 Cytotoxicity study on the Caco-2 cell model

MTT Assay.

The developed probiotic beverage sample was tested for *in vitro* cytotoxicity, using Caco2 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, the cultured Caco2 cells were harvested by trypsinization, pooled

in a 15mL tube. Then, the cells were plated at a density of $1\times10_5$ cells/mL cells/well (200 µl) into a 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the M sample in a serum-free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µl of 5 mg/mL) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µl) were aspirated off the 37 wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µl) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC_{50} value was calculated using Graph Pad Prism 6.0 software (USA).

Assay controls.

(i) Medium control (medium without cells) -Blank

(ii) Negative control (medium with cells but without the experimental drug/compound).

Sample preparation and experimental groups.

A 2mg/mL stock solution of Ferrous tablet was prepared in 0.01N Hcl and used as an iron source. Experimental blank contains only 0.01N Hcl and Ascorbic acid with 50ug/mL and Sample V_1 with 50ug/mL was used. Fe + AA mixture contained 2 mg Fe and AA equivalent of V_1. Fe + V_1 mixture contained 2mg Fe and V_1. All the groups were prepared in 15mL screw cap sterile centrifuge tubes.

Determination of IC_{50} value.

According to the FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition in vitro. IC_{50} is a concentration of drug at which 50 % of the cell population dies. The 50 % of cell growth inhibition was used as the cut-off for compound toxicity against cell lines. IC_{50} values were determined from the plot of the dose-response curve between concentration and percentage inhibition of cell growth. IC_{50} values were estimated as a concentration of the drug at 50 % position on the Y axis.

Assessment of cell morphology.

Phase contrast microscopy was used to assess the morphological changes in Caco-2 cells after treatment with the beverage. Phase contrast makes living, unstained microscopic structures visible. This is useful for specimens that possess little or no colour of their own and which have not been artificially coloured such as living biological specimens, cells, and organ cultures. Cultured cells were treated with different concentrations of the beverage. Plates were incubated at 37°C in a CO_2 incubator with 5% CO_2 for 48 h.

3.5.4 Iron Uptake study in Caco-2 cell model.

The test compound (Fe + K) and control dialysates (Fe/AA/ K alone and blank) were used to study the iron uptake in Caco-2 cells. The protocol described by Glahn *et al* with modifications as described by Venkatasubramanian *et al* was followed for iron uptake studies. Briefly, 0.25 x 10^6 cells/mL of Caco-2 were cultured in a 6-well plate and incubated and maintained for 14 days by replacing the media every 3 days once. After 14 days, wash the cells with 1x HBSS buffer and add 1 mL of DMEM medium alone. Treat the cells with dialysates of the required test groups and incubate the plate for 24 hours at 37°C with a 5% CO_2 atmosphere. Further cells were solubilized in 0.5 mol/l NaOH (500µl per well) harvested the cells by trypsinization and collected in a 2 mL fresh microcentrifuge tube. Homogenize the cells by repeated thawing steps, centrifuge at 2500 rpm for 5mins, and collect the supernatant into a fresh sterile microcentrifuge tube. The total protein in the supernatant was estimated by Bradford assay. The amount of ferritin in cell lysate was estimated by Raybio Ferritin ELISA kit (Ray Biotech, Norcross, GA) following kit protocol and expressed in terms of ng/mL.

Concentrations

In this study, the concentrations of the different conditions used to treat the cells are depicted in Table 3.2.

Table 3.2: Details of drug treatment to respective cell lines used for the study.

Sl. No	Culture condition	Cell lines	Concentration treated to cells
1	Untreated	Caco-2	No treatment
2	Blank	-	Only Media without cells
3	Fe	Caco-2	2mg/mL
4	V1 - 50ug	Caco-2	50ug/mL
5	Iron+Ascorbic acid (AA)-50ug	Caco-2	2mg/mL+50ug/mL
6	V_1+AA -50ug	Caco-2	2mg/mL+50ug/mL

PHASE VI

3.6 Statistical analysis and interpretation of the study outcome.

Statistics were used to analyze the relationship between different formulations and other parameters. The data generated from the analysis were presented in tables, graphs, etc. The statistical methods used in the present investigation are given below. Results are presented as means of two or three independent determinations and expressed along with standard deviations. Standard deviation was determined using Microsoft Excel (2010) software. Significant differences were declared at $p \leq 0.05$ and $p \leq 0.01$. The following were the statistical tests applied in the different phases (Appendix III).

Descriptive Statistics

The descriptive statistics viz., mean and standard deviation (SD) were used to evaluate the macronutrient, micronutrient and heavy metal profile of the selected edible seaweeds, analysis of physical characteristics and nutritional parameters of the developed probiotic beverage, depiction of phytochemical profile of the developed beverage, *in vitro* antioxidant potential, antibiotic activity of probiotic strain on common enteropathogens, the activity of the beverage on common enteropathogens and ferritin concentration of the Caco-2 cells during iron bioavailability study.

Data were analyzed by SPSS statistical software and means for bacterial growth after different treatments were compared by the independent sample Student's *t*-test. The comparison of the percentage of coaggregation between isolates and over time with pathogens was performed using a paired-sample *t*-test.

Correlation Studies

A Pearson‘s coefficient correlation was run to determine the correlation between auto aggregation and hydrophobicity percentages. The correlation between DPPH RSA, FRAP, Total Phenols, Total Flavonoids, Alkaloids and oxalates was successfully established. Also, correlation was established between sensory attributes of the beverage with total phenols and flavonoids, to determine the associations.

Percentage Calculation

The percentage was calculated for parameters including Free Radical Scavenging Activity (RSA) of the probiotic beverage, cell surface hydrophobicity and auto-regression assay, NaCl tolerance test, viability of the cells during cytotoxicity study, ferritin uptake by caco-2 cells. Ratios and indices were determined to assess the nutrient-antinutrient interaction and nutrient quality score.

Paired T- test

Paired t-test was applied to analyze pH, gastric acid tolerance, bile tolerance and pancreatin tolerance.

ANOVA and Post-hoc Tukey’s test

ANOVA and post-hoc Tukey‘s test was done to distinguish significant differences among the organoleptic scores of each parameter used to assess the sensory acceptability of the developed probiotic beverage.

Data Mining and Analysis.

Agilent Personal Compound Database and Library (PCDL) was utilized to generate a database with the phenolic compounds obtained from ChemSpider (http://www.chemspider.com/), Phenol-Explorer (http://phenol-explorer.eu/), PubChem (https://pubchem.ncbi.nlm.nih.gov/) and FoodB (https://foodb.ca/). Drug-like properties was done using QikProp module from Schrödinger software suite, Maestro 11.0 (LLC Schrodinger 2016.

3.7 Conceptual Framework of the Study.

The conceptual framework in research provides a systematic and organized approach to delineating the relationships between key variables, thereby guiding the formulation of

research objectives and hypotheses. It articulates the theoretical underpinnings, constructs, and assumptions that inform the study. Creswell *et al.,* (2016), a robust conceptual framework aids in identifying and defining critical variables and their interrelationships, thus providing a clear direction for hypothesis development and data collection. Grant *et al.,* (2017) further asserted that a well-constructed framework facilitates the identification of research gaps, ensuring the relevance and precision of the study ‘s focus. In the context of developing an *Ulva lactuca* based probiotic beverage, this framework would encompass the biological, biochemical, and technological dimensions that influence the product ‘s effectiveness and its potential impact on bioavailability. By integrating these variables, the framework serves as a foundational tool for exploring the functional properties of *Ulva lactuca* and its interaction with probiotics, thereby enhancing the scientific rigor of the investigation.

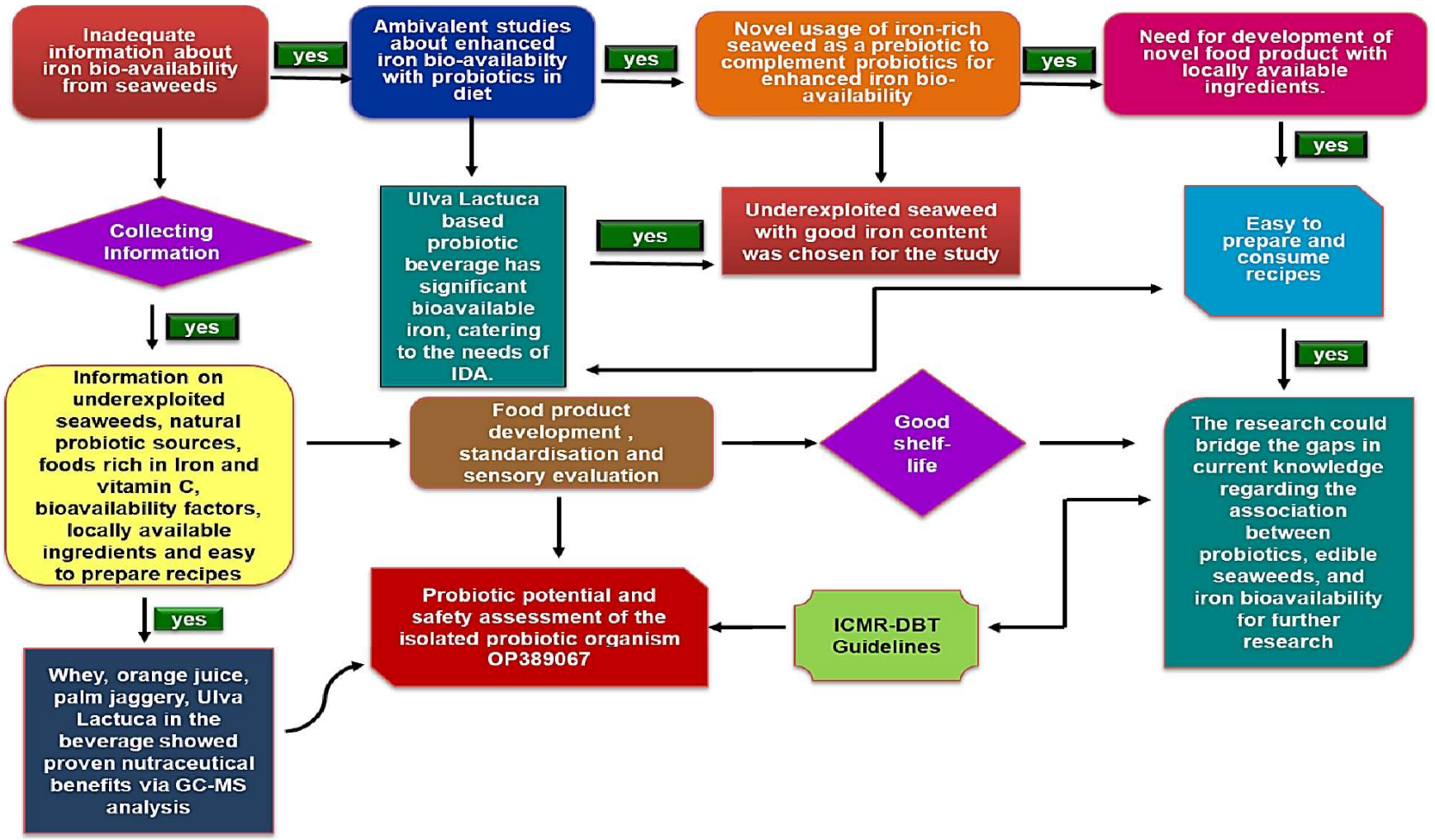

Fig. 3.5. Conceptual framework of the study