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Design and Evaluation of Enteric-Coated Synbiotic Systems for Enhanced Probiotic Using Novel Prebiotic Delivery

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ABSTRACT

Design of a delivery systems that release probiotics specifically in the colon to maximize their therapeutic effects a targeted approach can potentially improve therapeutic outcomes compared to systemic or less specific delivery methods. An attempt was made to design and develop a synbiotic delivery system by using Eudragit L-100 and Eudragit E-100 as coating materials to enhance the viability of probiotic cells in the core. Prebiotic material was extracted from banana bract, agave leaves, and orange peel. After evaluation, prebiotic potency was evaluated positively with all three extracts against *Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacteria bifidum*, and *Bifidobacteria lactis.* Lactobacillus strains showed a positive response and were thus used for the formulation development. Cell viability after formulation, and cell release in the gastric and intestinal pH showed positive outcomes. In vivo, studies can be performed as part of the future scope of the present research work.

KEYWORD: Synbiotic, Prebiotic, Probiotic, Enteric coating

1. INTRODUCTION

Gibson and Roberfroid introduced the term "synbiotic" for the first time in 1995. A synbiotic is a combination of probiotics and prebiotics which act together synergistically to improve the health benefits of consumer.[1] Probiotics are live-beneficial bacteria that contribute to the health of the gut microbiome, while prebiotics are non-digestible food ingredients that selectively stimulate the growth and/or activity of these beneficial bacteria. Combining both, synbiotics aim to improve the survival and colonization of probiotics in the gastrointestinal tract, providing a more significant positive impact on the host's health than either component alone.[1] According to the Food and Agriculture Organization/World Health Organization guidelines, probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host'.[2] The term "probiotic" was first used in 1965, by Lilly and Stillwell, to describe substances secreted by one organism which stimulate the growth of another.

Nowadays, probiotics represent an important group of beneficial consumed/supplemental microorganisms that can live in foods/supplements and the intestine.[2]. Benefit of probiotics administration has reported for many illnesses like, chronic bowel syndrome, ulcerative colitis, glycemia, reducing LDL, allergies, immunologic disorders, diabetes, genitourinary infections, ovarian diseases etc. The gut microbiota, comprising trillions of microorganisms inhabiting the gastrointestinal tract, plays a pivotal role in host metabolism, immune modulation, and gut barrier function.[3] Probiotics are mainly bacterial strains of Lactobacillus, Bifidobacterium, and Enterococcus and the yeast Saccharomyces.[4,5]

The zero risk does not exist, and acceptance of the concept that probiotics may not only have positive effects but potentially, also side effects is important. Mild side effects from probiotics such as bloating, constipation, flatulence, dyspepsia, nausea, and abdominal discomfort rather do not influence the safety of the probiotic. due to the rapid emergence of probiotics as drugs, a harmonized approval process similar to the other drugs, covering all aspects of Investigational New Drug Application (INDA) and New Drug Application (NDA) has been proposed in which organisms falling under Generally Recognized As Safe (GRAS) category are exempted from INDA submission whereas, non-GRAS, GRAE or new organisms are not exempted. [6–8].

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health. They serve as a food source for beneficial gut microorganisms, such as bifidobacteria and lactobacilli, promoting a healthier gut microbiome. Prebiotics can be found in various foods, including onions, garlic, bananas, and whole grains. They are distinct from dietary fiber because they specifically enhance the growth of beneficial bacteria. [1,4,9–11]

2. MATERIALS & METHODS

2.1.Prebiotic Material

The inflorescences from *Musa paradisiaca*, were obtained from a farm in Pimpri, Pune. It was authenticated with voucher specimen no. SOMUS02 deposited in Botanical Survey of India, Pune during past study. The leaves of *Agave americana* plant were obtained from a garden at Pimpri, Pune. It was identified and voucher specimens no. SOAAS01 have been deposited in Botanical Survey of India, (BSI) Pune during past study. The ripe fruits of orange i.e. *Citrus reticulata* were collected from the Nagpur region. Fruits were identified and peeled to separate yellow portion and dried in shed.

Proximate analysis of all the crude drug powders was carried out by quantitative determination of moisture content, total ash, acid soluble ash values, extractive values according to standard procedure of Indian Pharmacopoeia. Total carbohydrate content was determined using the phenol sulfuric acid assay[12]. Bradford's assay was used to determine the concentration of a solubilized protein[13]. The total phenolic content of the crude drugs was determined using the Folin-Ciocalteau method.[14,15] The total flavonoid content of the crude drugs was determined using a modified colorimetric method.[15]

The inflorescences from the authenticated banana plant were cut from the stems after the last *Musa paradisiaca* comb was produced and within three consecutive bracts discarded. The whole inflorescence was washed thoroughly under running water and shed dried. These were chopped into small pieces and dried in a hot air oven at 70°C. The dried pieces (approximately

80-90 g) were ground coarsely with a blender and stored in a desiccator for further use. Dried *Musa paradisiaca* inflorescence coarse powder was defatted with petroleum ether with subsequent drying and maceration in 500 mL of absolute ethanol with intermittent agitation for 72 hours at room temperature. The ethanolic extract was filtered through a Whatman No. 1 filter paper, and the residue was reextracted with ethanol until the extract became colourless. Each filtrate was combined and evaporated to reduce the volume. The final *M. paradesica* ethanolic extract (MPE) and residue of the inflorescence was oven-dried and both were kept at -20°C for future use.

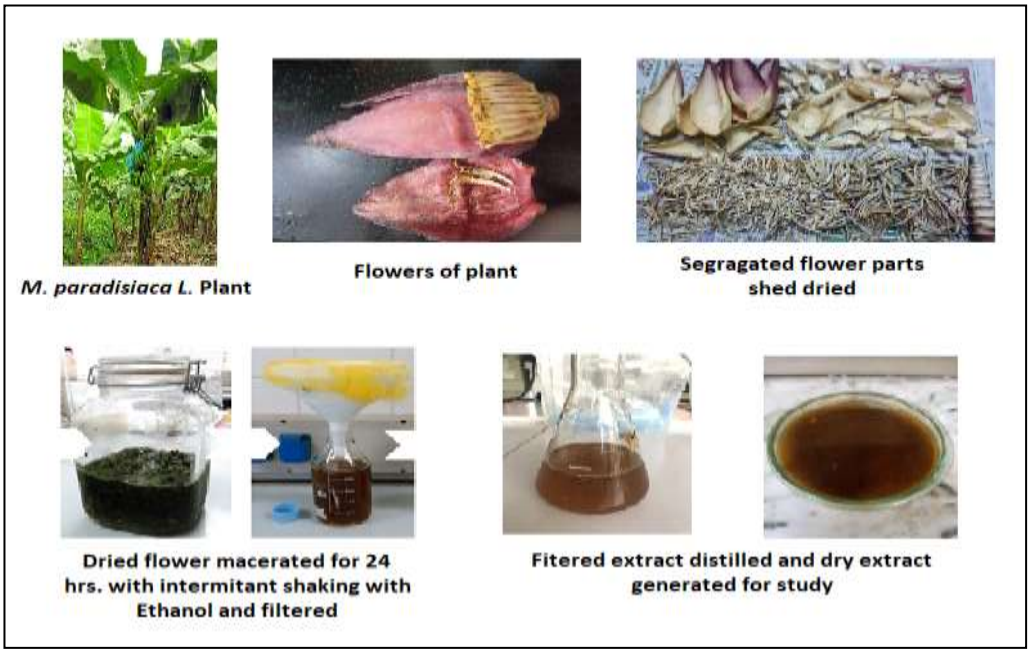

Figure 1:M. Paradesiaca L. Extraction process

All the leaves of Agave were washed thoroughly under running water and shed dried. These were chopped into small pieces and dried in a hot air oven at 70°C. The dried pieces (approximately 80-90 g) were ground coarsely with a blender and stored in a desiccator for further use.

Dried *A. americana* leaves coarse powder was defatted with petroleum ether with subsequent drying and maceration in 500 mL of absolute ethanol with intermittent agitation for 72 hours at room temperature. The soluble sugars were extracted with 15 mL of 96% ethanol and then centrifuged at 10,000 × g for 20 min at 4°C. The extract was filtered through a Whatman no. 1 filter paper, and the residue was reextracted with ethanol until the extract became colourless. Each filtrate was combined and evaporated to reduce the volume. The final *A. americana* (AAE) and residue was oven-dried and both were kept at -20°C for future use.

Citrus reticulata i.e. Nagpur oranges we collected from local market in Nagpur in the month of December 2021. The fruits were physically examined to ascertain their wholesomeness and washed with large quantity of water to remove the glycosides the bitter taste of the peels. Peels were separated and cut into smaller pieces for easy drying and then dried using tray drier for 2.5 to 3 hr at 90°C. The dried peels were powdered by using grinder and stored at in air tight container until use. 100 g of the peel powder was blended 1000 ml distilled water and added

with 2.5 ml hydrochloric acid dropwise to maintain pH of 1.5. It was heated at 94°C for 1 hr with intermittent stirring with glass rod. Hot mixture was rapidly cooled to 38°C using ice water bath and filtered under vacuum [11]. Pectin containing aqueous extract was coagulated by using an equal volume of 99% ethanol at 4°C and was left for 18 hrs. The floating pectin was separated and washed again with 99% ethanol. It was then conditioned in small cloth bags and immersed in acetone for approximately 15 hrs for the partial removal of the acid. The pectins were dried in a drying cabinet with air-circulation at 40°C for approximately 5 hrs, till a constant weight was achieved. achieved (moisture between 8 and 10 %). Samples were ground, homogenized and sieved in order to obtain powdered pectin. It was then stored in desiccators until further use. Percent yield of was calculated.

Extracted and purified products including BBE, AAE, and OP were investigated for the parameters like yield, physiochemical characteristics, proximate analysis etc.[16] Extraction yield of isolated compounds from raw material was also determined using following equation.

$$Yeild = \frac{Weight\ of\ dried\ product\ (g)}{Weight\ of\ dried\ crude\ material\ taken\ for\ extraction\ (g)} \times 100$$

---------- *Equation 1*

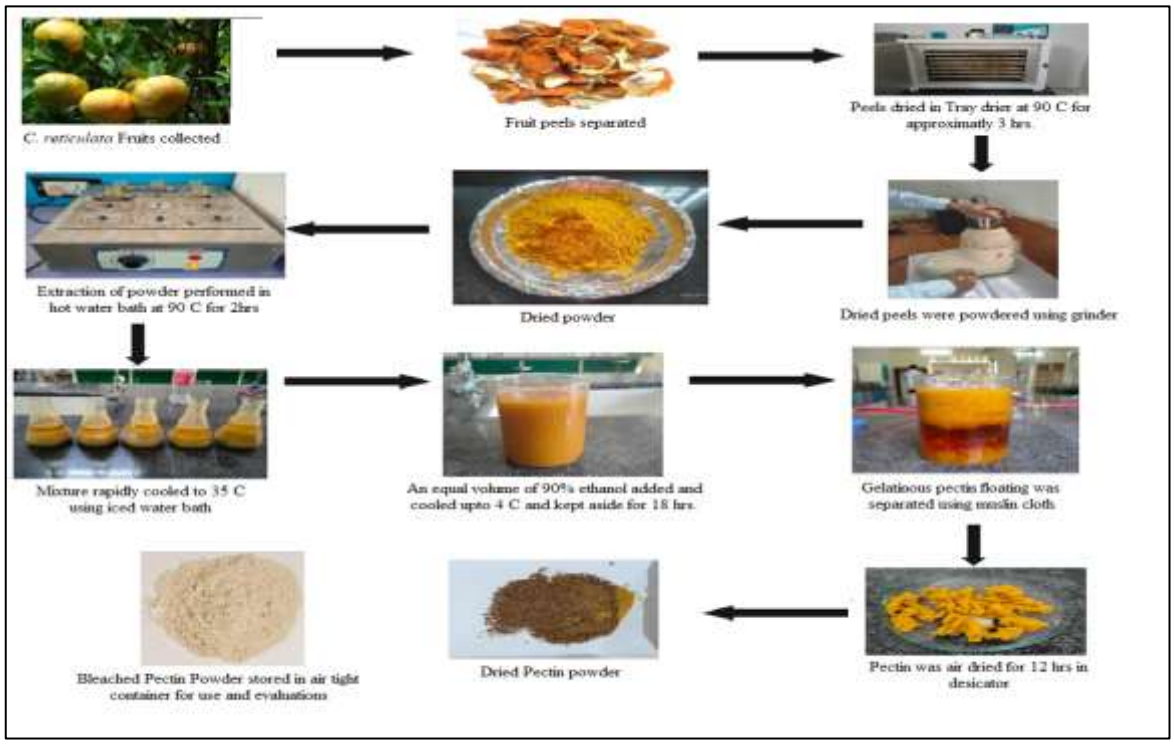

Figure 2: C. reticulata L. Extraction process

Physiochemical Properties were evaluated including morphological characteristics like appearance of the extracted products were studied for characteristics like colour, texture, consistency. Swelling power, solubility was also studied in different solvents. Preliminary phytochemical screening was performed for all the extracts for the presence of carbohydrates, proteins, starch, glycosides, saponins, alkaloids, flavonoids, tannins, content was performed for all the extracts.

2.2.Procurement and Preparation of Probiotic Material

Lyophilised Probiotic bacterial strains *of L. acidophilus* (LA)*, L. plantarum* (LP)*, B. bifidum* (BB)*,* and *B. lactis* (BL), kindly provided by Unique Biotech Pvt. Ltd, were used as probiotic model bacteria in the experiment described. The number of probiotic cells in these lyophilised granules ranged from 10^{10} to 10^{11} CFU/g. These cells were revived using de Man Rogosa and Sharpe (MRS) Media by suspending 1 gm of each cell separately in the test tubes containing 10 mL of MRS media. Each of these sample was serially diluted with 9 mL of MRS media in separate test tubes. Each of these sample then poured in sterilised petri plates and incubated at 37°C under anaerobic conditions for 24 Hrs. for analysis purpose. Whole experiment was performed aseptically. Colonies grown in the range 30 to 300 were observed and colony forming unit per gram was calculated. A prominent colony from each selected plate was isolated aseptically and inoculated on the freshly prepared sterilised MRS media slant. It is stored and sub-cultured weekly for assuring regular supply for analytical studies during project in MRS broth and stored in refrigerator at -20°C.

Procured strains were revived and characterised for confirmation of strains. [17–20] The isolated colonies formed on the de Man Rogosa and Sharpe (MRS) agar (Hi-media Pvt. Ltd.) plates were identified phenotypically for colony characteristics, spore formation, Gram staining, lactic acid fermentation test, catalase test, carbohydrate test and arginine tests. The identification was performed according to Bergey's manual of determinative of bacteriology. The culture was kept in MRS agar slant and stored at 4^0C. For long term storage, glycerol stocks were maintained and stored at 20^0C. Phenotypic characteristics, microscopy, Gram staining, spore formation test was performed. Biochemical tests are essential tools in microbiology for identifying and characterizing bacteria based on their metabolic activities. Some commonly used biochemical tests for identification of lactobacilli and bifidobacteria include lactic acid fermentation, catalase test, carbohydrate fermentation test, acid production test, arginine hydrolysis test, and gas production test.

2.3.In-vitro Prebiotic potential determination

2.3.1. Suitability testing for media to be used for prebiotic studies

Optimal Basal media was identified to evaluate the suitability for diluting the prebiotics material to study prebiotic potential on the growth of the selected probiotic strains.[21] MRS medium deficient in any direct carbon source (basal M) and RCM medium deficient in any direct carbon source (basal R) were assessed for the purpose. 100 mL of MRS and RCM media was added with a concentration of 0.5% glucose as the sole carbon source and autoclaved for 15 min at 121°C. Following media preparation, a 1% inoculum of an overnight culture ($1x10^9$ CFU per ml) of each probiotic bacterial strain was added separately in each media tube, and this was incubated in micro-aerophilic conditions overnight at 37°C. Growth of each strain was monitored after 24 hrs, by measuring the optical density (OD) of the cultures at wavelength of 600 nm using a UV spectrophotometer.

2.3.2. Test Tube Method

Growth of the probiotic strains was monitored according to modified procedure mentioned by Ping Su,[21] in a basal medium supplemented with the test prebiotic BBE, AAE, and OP at a concentration of 1 %, as the sole carbon source. To prepare the medium, the BBE was first dissolved in basal broth then autoclaved for 15 min. at 110°C. Based on the results of the above preliminary studies, MRS i. e. basal M media was used for LA and LP, while RCM i. e. basal R was used for BB and BP. Following media preparation, a 4% prewashed inoculum of a given probiotic overnight culture ($1x10^9$ CFU/ml) was used to examine the effect of the pre-determined prebiotics on the growth of the probiotic culture. Cultures (200 mL per cultivation) were incubated in micro-aerophilic conditions at 37°C for a period of 48 hrs. under non-pH-controlled conditions. Growth of each strain was monitored by measuring the optical density (OD) of the cultures at 0, 2, 4, 6, 8, 10, 12, 24, 28, 36 and 48 hrs. at wavelength of 600 nm. Measured values of optical density were plotted on growth curves. The growth rate was calculated during the exponential growth phase through the following equation. [22]

$$\mu max = \frac{In(ODmax) - In\ (ODmin)}{T} \qquad \text{-------}Equation\ 2$$

Where,
OD_{max}.: OD at the end of exponential growth phase
OD_{min}.: OD at the beginning of exponential growth phase
T: Time interval between observations.

The pH was measured by an electronic digital type pH meter. pH 4.0 and pH 7.0 buffer solutions were used to standardize the pH meter. Measured pH values were plotted on graphs.

2.3.3. Validation through Plate count methods

Inoculum containing probiotic bacterial strains and extracts were separately diluted with Phosphate Buffer Solution (PBS) (Hi-media Pvt. Ltd.) and plated on MRS & RCM plates supplemented with 1.5% agar (Hi-media Pvt. Ltd.). The plates were incubated in anaerobic chamber at 37°C and enumerated after 3 days. **(**Sampo J. Lahtinen, 2006) Colonies were identified by colony morphology and the identification was confirmed by biochemical tests. Colonies were enumerated and the results were expressed as colony forming units per gram of stool. Re-characterization The Bacterial colonies grown along with the prebiotic material were observed for phenotypic characteristics like shape of colony, colour, margin, hight, and diameter. Colonies were isolated separately in the MRS broth for strain identification. The isolated colonies were stained separately for Gram's staining and observed under microscope under 10x and then 100x oil immersion lens of compound light microscope. The phenotypic characteristics like shape of the cells, cell arrangements, presence of flagella were observed to confirm the type of Bacteria. Biochemical tests were performed on the basis of carbohydrate fermentation test, motility test, catalase and oxidase test, for confirming type of probiotics strains grown.

Synbiotic blends containing all probiotic strains of probiotic bacteria evaluated in previous chapter. Optimised synbiotic blend of probiotic strains *L. acidophilus* (LA)*, L. plantarum* (LP)*,* and prebiotic material i.e. BBE, AAE, and OP was considered for the development of enteric

coated formulation, which should release the active content in colon for its desired therapeutic benefit.

2.4.Pre-Formulation Studies

The prebiotic materials i.e. BBE, AAE, and PO are evaluated for the important parameters which may interfere the integrity and stability of formulation for effective delivery. Hygroscopicity [23,24] solubility index in distilled water, 0.9% NaCl, 0.1M HCl, and 0.1M NaOH, all at room temperature, as well as at pH 7.4 phosphate buffer at 37°C.Each sample was evaluated in triplicate. Mean was calculated using following equation. Bulk density, tapped density, angle of repose, Car's compressibility index was performed according to standard methods. Compatibility studies were performed for each prebiotic sample i.e. BE, AAE and OP are mixed with each other and with the excipients i.e. magnesium stearate and croscarmellose in the ratio 1:1 and triturated with KBR to make a pellet for IR spectra. IR spectra was compared to find any interactions.

2.5.Formulation of Granules

All the ingredients were blended in a sequence projected in above figure. Lyophilised probiotic bacterial strains i.e. LA and LP was mixed in the equal quantity and subsequently with AAE in the ratio 1:1 to form a Synbiotic blend I. BBE was mixed in the 10 % w/w of total weight of Synbiotic blend I and referred as Synbiotic blend II. OP was mixed in varying quantity according to formulation requirement, as it may interfere the disintegration or compressibility of the granules. Final blend is referred as Synbiotic blend III, was passed through 300µm diameter sieve to breakdown any lumps and form a uniform powder. These powders are evaluated for flow properties loose bulk density, tapped bulk density, angle of repose, Hausner's ratio, Car's compressibility Index, as mentioned above to develop the formulation granules. Sample of Synbiotic blend III was also evaluated for solubility and stability in the same manner explained in previous section. The optimised formulation experimentation is presented in the following flow chart.

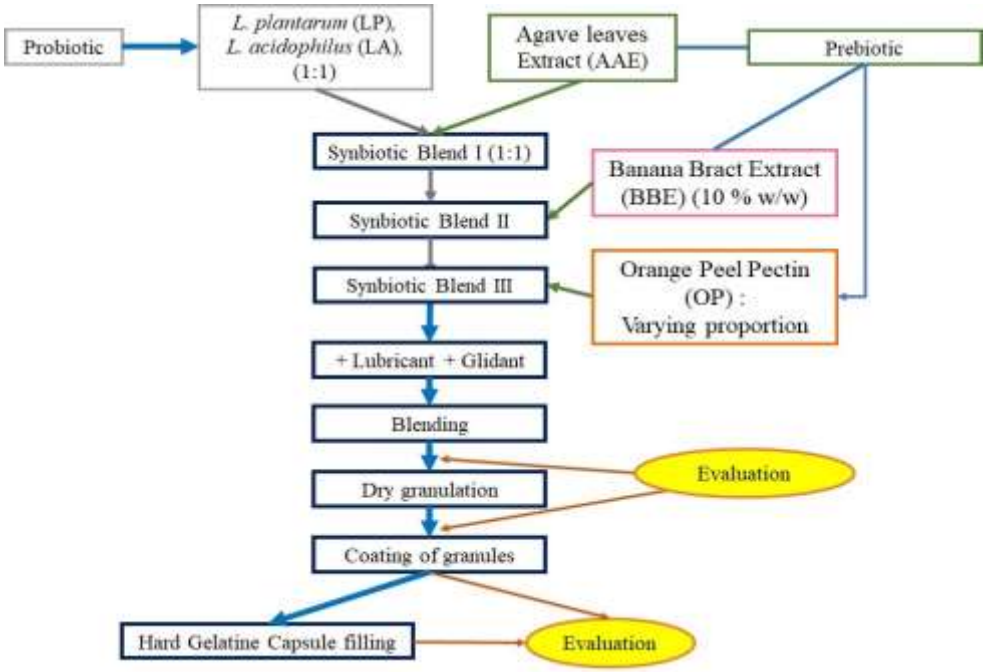

Figure 3: Process flow of the formulation optimisation

Compression granulation also called as dry granulation has been used for many years, and is a valuable technique in situations where the effective dose of a drug is too high for direct compaction, and the drug is sensitive to heat, moisture, or both, which precludes wet granulation. Therefore, this technique was used to avoid interference of moisture with the characteristics of excipients, especially pectin and also to avoid precision of probiotic cells count. Agave extract and pectin functions as diluent and binder for developing a core. These ingredients along with Croscarmellose sodium as disintegrant and magnesium stearate as lubricant are blended in composition as mentioned in table. The powders are blended using a planetary mixer (Kenwood, UK) for 5 min. The powder blend is forced into the dies of a large capacity punching machine with a flat faced die of 2 in diameter to form a slug. The slug so formed is milled to produce a granular form of tabletting material. Slugging process is repeated to get uniform granular mass.

Table 1: Compositions and Optimisation of formulation

Ingredient	**Function**	**Formulation Concentration as % w/w**		
		S1	**S2**	**S3**
L. plantarum (50%w/w)	Probiotic	45	45	45
L. acidophilus (50%w/w)				
Agave leaves extract	Prebiotic/Filler	45	45	45
Banana bract ethanolic extract	Prebiotic	6	6	6
Orange pectin	Prebiotic/Disintegrant	2.5	2	1.5
Mag. stearate	Lubricant	1	1	1
Croscarmellose sod.	Disintegrant	0.5	1	0.5
	Total Weight	100	100	100

Granules were prepared in different formulations viz. S1, S2, and S3. The formulation showing best results out of these was manufactured in bulk and considered for enteric coating. The design of the system is as shown in following figure.

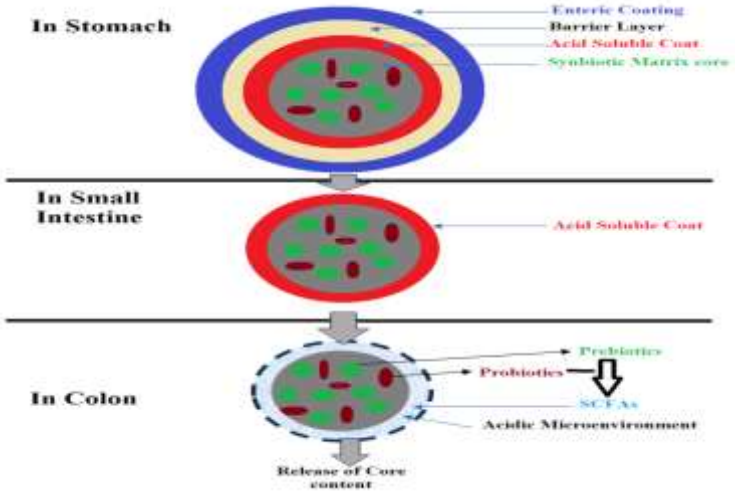

Figure 4: Design of Synbiotic delivery system to release in Colon.

Probiotic cells viability can be extended by protecting them in the gastric conditions through enteric coating. First layer next to the core granules was HPMC coat as a barrier, second layer was Eudragit E-100, third layer was HPMC barrier and the outer layer was Eudragit L-100. Composition of different coating solutions is given in the table 2.

Table 2: Composition of coating solutions

Sr. No.	Ingredients	Quantity
1. Acid soluble coating		
i)	Eudragit E-100	35 g
ii)	Methylene chloride	490 mL
iii)	Castor oil (plasticizer)	10 mL
2. Barrier layer coating		
i)	HPMC	10 g
ii)	Isopropyl alcohol	300 mL
iii)	Methylene chloride	200 mL
3. Enteric coating		
i)	Eudragit L-100	45 g
ii)	Methylene chloride	490 mL
iii)	Castor oil (plasticizer)	10 mL

Coating optimization was undertaken with reference to a prior research study by a specific author. The initial coat, adjacent to the core matrix granule, was optimized to 6% w/w. The secondary coat, functioning as a barrier to prevent interactions between the acid-soluble and enteric layers, was established at 2% w/w. The terminal coat, an enteric layer designed to provide gastric protection, was optimized to 4% w/w. [25]

To achieve suitable processing conditions for the top-spray fluidized bed coater in granule coating, different coating parameters were optimized, such as inlet temperature, fluidization, air flow rate, atomizer pressure and spray rate, using one-at-a-time strategy by varying one parameter each time while keeping the others constant. The optimised process parameter used for the coating of granules, which provides maximum viability and no agglomeration is mentioned in table 3. The granules were coated with three consecutive layers as mentioned in above table. Coated granules were allowed to fluidize for 10 min to ensure complete drying after spraying.

Table 3: Process parameters for the coating experiments

Sr. No.	Process Parameter	Parameter Used
1.	Coating nozzle diameter	1 mm
2.	Spraying rate	1 ml/min
3.	Pan Speed	15-20 RPM
4.	Inlet air temperature	35-38 °C
5.	Baffles	04
6.	Air Pressure	1 bar

Coated granules are the final formulation to be utilised for the therapeutics in polycystic ovarian syndrome. The are weighed and stored in tightly packed opaque container to avoid any stability issue and stored in refrigerator for further studies. 500 mg of coated granules were then filled manually into hard gelatine capsules of size zero.

2.7.Evaluation Granules

Coated granules are evaluated for the general appearance includes determination of granule size, shape, color, presence or absence of an odour, taste, surface texture, and any physical flaws. The coated granules were evaluated for various parameters as mentioned in this chapter to observe the physical properties, flow properties, swelling ratio, content release, viability of probiotic bacteria, pH alteration, disintegration and stability. Moreover, percent weight gain after coating and coat thickness is determined for the final coated granules formulation.

2.7.1. In- vitro Content Release Study of Optimised Formulation

The in vitro dissolutions of capsules filled with the formulated enteric coated synbiotic granules were studied under simulated gastric and intestinal fluids to study the release time. Simulated gastric fluid and intestinal fluid were prepared according to the method described in the United States Pharmacopoeia as mentioned in table 4.

The dissolution study of optimized colon specific drug delivery system was performed in different pH media by using USP basket dissolution apparatus (Type-I). Continuous supply of carbon dioxide was maintained during this study to maintain the anaerobic conditions as shown in the following picture.

For evaluation of cells viability in gastric and intestinal condition, 900 mL of dissolution medium i.e. SGF and SIF was maintained at 37.0 ± 0.5 °C in dissolution jar. 2 capsules were placed in backet and a rotation speed was set at 100 rpm. 5 mL sample was withdrawn after

each 30 min up to disappearance of granules observed. Same amount of dissolution medium is reconstituted on withdrawal. The release of bacterial cells from the capsule in dissolution medium was estimated using UV spectrophotometry and the pour plate method. The test was run in triplicate. pH changes also measured for every sample.

Table 4: Composition of dissolution media

Simulated gastric juice	
Sodium chloride	2.0 g
Purified pepsin	3.2 g
Hydrochloric acid	7.0 mL
Water	Up to 1000 mL
pH	1.1–1.2
Simulated intestinal fluid	
Monobasic potassium phosphate	6.8 g
0.2 mol L–1 sodium hydroxide	77 mL
Pancreatin	10.0 g
Water	Up to 1000 mL
pH	6.8 ± 0.1

The dissolution study of developed system was performed in pH 1.2 buffer solution for two hrs and drug release was calculated then formulation was transferred to pH 6.8 buffer solution and dissolution was carried for four hrs. The dissolution study showed only 10 % of content release at the end of six hrs which is as per the criterion for colon specific drug delivery system. The formulation was further evaluated in 6.8 pH buffer solution for next two and half hrs. The developed formulation does not show significant drug release i.e. only 39 % at the end of eight and half hr may be due to absence of ceacal content which is responsible for triggering the release from the system. The drug release from the developed system in colonic condition is dependent on interaction between Saccharide and colonic microflora. The interaction between Saccharide and colonic microflora results in acidic microenvironment which dissolves the Eudragit E-100 layer, as Eudragit E-100 polymer solubilized only in acidic environment. The developed system showed maximum release in colonic condition. The system triggered the release in presence of colonic microflora in core matrix of granules, which also revealed the system's site-specific characteristic.

Figure 5: Dissolution apparatus with carbon di oxide supplimentation during content release study

2.8.Measurement of the Probiotic Viability

The viability of LA and LP in the prepared granules was expressed as colony forming units per gram sample of granules (CFU/gm). The weighed granules were then placed into a sterile tube containing a sterile diluent, such as 9 mL of phosphate-buffered saline (PBS), to create an initial 1:10 dilution. The mixture was homogenized to disaggregate the granules and ensure an even suspension of bacterial cells. Following this, serial dilutions were performed by taking 1 mL of the initial suspension and adding it to 9 mL of sterile diluent to create a 1:10 dilution, continuing this process to achieve further dilutions. (e.g., 10^{-2}, 10^{-3}, 10^{-4}).

Next, 100 µL of each dilution was plated onto separate sterile MRC media containing petri plates, and the sample was spread evenly across the surface using a sterile spreader or L-shaped rod. Control plates with just the diluent i.e. PBS were included to check for contamination. These plates were incubated at an appropriate temperature for the bacterial species under study, typically at 37°C for 24-48 hours, until colonies were visible and countable. After incubation, colonies on plates that had between 30 and 300 colonies were counted, as plates with more than 300 colonies were considered too numerous to count (TNTC) and less than 30 may not have been statistically significant. CFU per gram of the original granule sample using the following equation:

$$CFU/g = \frac{(No.\,of\ colonies\ \times Dilution\ factor}{(Volume\ plated\ in\ mL \times Weight\ of\ granules\ in\ gm)}$$

2.9.Stability Study of powders

To determine the solid-state stability profile of powders, 5 mg of each sample is placed in open screw-cap vials and are exposed directly to a variety of temperatures, and humidities as shown in following table. Samples were analysed at the interval of 15 days weeks up to 3 months. Each sample was kept in triplicate. Room temperature with ambient humidity condition was selected for the storage of formulation as mentioned in the following table. After a fixed

exposure time, these samples are removed and analysed by for general appearance, and cell viability.

3. RESULTS AND DISCUSSION:

3.1. Pharmacognostic evaluation of Prebiotic Materials

The banana bract is a modified leaf that serves to protect the developing fruits and flowers on the banana plant's flower stalk. These bracts are large, ovate, and fleshy, measuring between 15 and 20 cm in length. The base of a bract is typically around 6.6 cm wide, while the middle can reach a width of 9 cm. In terms of appearance, banana bracts are usually purple or red, though they can also feature yellow at the tips and outer surfaces. Structurally, they are convolute, meaning their tips overlap in a manner that provides additional protection to the young fruits and flowers within.

Agave leaves are up to six feet long and 10 inches wide, sword-shaped and arranged tight rosette form on plant. These are blue green in colour. The leaves of Agave americana are large, rigid, and sword-shaped, typically measuring between 1.2 to 2.4 meters in length and 15 to 20 cm in width. They exhibit a blue-grey to grey-green coloration and possess sharp spines along the edges with a pronounced pointed tip. The leaf margins are adorned with small, recurved teeth, contributing to their distinctive appearance. The leaves grow in a basal rosette formation, with new leaves emerging from the center as the plant mature.

The peel of Citrus reticulata, or mandarin orange, presents distinct morphological attributes. It is relatively thin and exhibits a smooth to slightly rugose texture, facilitating ease of dehiscence in comparison to congeneric citrus species. The peel displays a chromatic spectrum from vivid orange to sporadically reddish or yellowish tinges, intensifying during fruit maturation. Its prominent feature is its olfactory richness, emanating a fragrant citrus aroma attributed to the profusion of oil glands distributed across its surface. Structurally, the peel comprises an external flavedo, characterized by its richness in essential oils, juxtaposed with an internal albedo, which is comparatively slender, spongiform, and pallid. This mesocarp's reduced prominence aids in the facile detachment of the peel from the fruit.

In the microscopic analysis of the bract isolated from flowers of *Musa paradisiaca*, commonly known as banana, several distinguishing characteristics are observable. The outermost layer, the epidermis, comprises closely arranged cells, often with a smooth surface and occasional stomata for gas exchange. Beneath the epidermis lies the mesophyll, consisting of palisade and spongy parenchyma. The palisade parenchyma consists of elongated cells arranged parallel to the leaf surface, optimizing light absorption for photosynthesis. Meanwhile, the spongy parenchyma contains loosely packed cells interspersed with air spaces, facilitating gas exchange and storage of water and nutrients. Vascular bundles, containing xylem and phloem tissues, traverse the mesophyll, enabling the transport of water, minerals, and sugars throughout the bract. Additionally, specialized structures such as oil glands or secretory cells may be present, contributing to the bract's unique functions, such as defence or attraction of pollinators. This microscopic examination offers insights into the anatomical adaptations of Musa paradisiaca bracts, elucidating their physiological processes and ecological significance in the reproductive cycle of the banana plant.

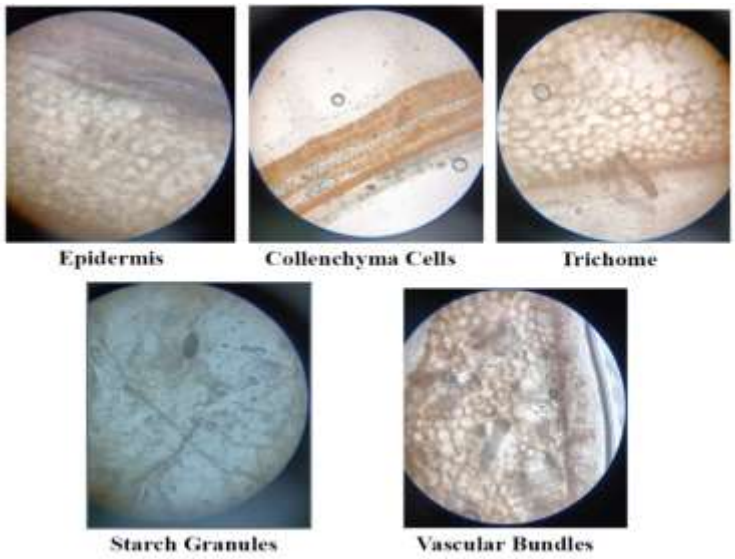

Figure 6: Microscopy of A. americana

In the microscopic examination of *Agave americana* leaves, several notable features emerge. The epidermis, comprising the outermost layer, consists of tightly packed cells, often exhibiting a rectangular or irregular shape and possessing stomata for gas exchange. Beneath the epidermis lies the mesophyll, characterized by two distinct layers: the palisade parenchyma, composed of elongated cells arranged parallel to the leaf surface, and the spongy parenchyma, consisting of loosely arranged cells with numerous intercellular spaces. The mesophyll houses chloroplasts, responsible for photosynthesis, and vascular bundles, facilitating the transport of water, nutrients, and sugars throughout the leaf. Additionally, specialized structures such as trichomes, which may be present on the leaf surface, contribute to defence mechanisms or regulate moisture levels. This microscopic examination provides insights into the anatomical adaptations of Agave americana leaves to arid environments, shedding light on their physiological processes and ecological roles.

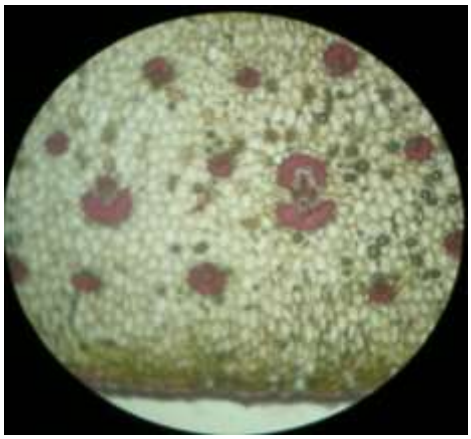

Figure 7: Transverse section of leaf tip portion of A. americana

In the microscopical scrutiny of orange peel, discernible features emerge. The epidermis, constituting the outermost stratum, comprises densely arranged cells, often exhibiting rectangular or polygonal morphology and accommodating numerous stomata for gas exchange.

Subjacent to this, the flavedo, characterized by its conspicuous chromaticity, harbours copious oil glands, discernible as diminutive, spherical entities ensconced within the parenchymal matrix. These oil glands are pivotal in imparting the distinctive olfactory profile to the peel. Predominating the tissue are parenchymal cells, expansive in size and serving as reservoirs for water, saccharides, and assorted nutrients. Dispersed amidst the parenchymal continuum are vascular bundles, instrumental in facilitating fluid and nutrient transport. Furthermore, specialized oil ducts contribute to the dispersal of essential oils throughout the tissue.

Figure 8: Orange peel upper epidermis under light microscope

3.2.Proximate analysis

The raw materials were dried in shade and powdered using a grinder for the proximate analysis as per Indian Herbal Pharmacopoeia. This analysis included determination of moisture content, total ash value, acid-insoluble ash value and extractive values of the crude drug.

Total carbohydrate contents, total phenolic content, alkaloid content and flavonoid content was determined using UV spectrophotometry. Calibration curves were obtained using standard compound in five subsequent concentrations. The unknown concentration is calculated using this calibration curve. Glucose, gallic acid, brucine, and quercetin is used to obtain calibration curve for carbohydrates, phenols, alkaloids and flavonoid respectively. The resultant values are represented in table 5.

Table 5: Proximate analysis of raw plant powders

Parameter	BEE	AAE	OP
Moisture content (%w/w)	4.00 ± 0.04	4.38 ± 0.03	0.60 ± 0.11
Total Ash Value(%w/w)	2.00 ± 0.02	2.94 ± 0.12	2.52 ± 0.05
Water Soluble Ash Value (%w/w)	12.00 ± 0.05	1.24 ± 0.05	0.98 ± 0.1
Acid Insoluble Ash value (%w/w)	3.96 ± 0.04	2.07 ± 0.02	1.87 ± 0.05
Water Soluble Extractive Value (%w/w)	2.00 ± 0.01	4.02 ± 0.01	1.20 ± 0.05
Alcohol Soluble Extractive Value (%w/w)	0.75 ± 0.01	0.22 ± 0.05	1.02 ± 0.05
Petroleum Ether Soluble Extractive Value (%w/w)	0.06 ± 0.02	0.07 ± 0.04	0.12 ± 0.02
Lipid Content (%w/w)	0.08 ± 0.07	2.03± 0.06	30.00 ± 0.04
Total carbohydrate determination (%w/w)	2.67± 0.04	94.70 ± 0.3	76.00 ± 0.01

Soluble sugar (%w/w)	0.17 ± 0.01	42.67 ± 0.74	29.42± 0.74
Insoluble sugar (%w/w)	0.80 ± 0.06	3.16± 0.63	48.00 ± 0.02
Protein (%w/w)	0.90 ± 0.05	3.45 ± 0.45	0.04 ± 0.04
Insoluble fibres (%w/w)	0.08 ± 0.05	29.37± 0.59	0.03 ± 0.05
Total protein (%w/w)	1.22 ± 0.05	35.33 ± 0.73	0.08 ± 0.04
Fat (%w/w)	0.01 ± 0.001	0.39 ± 0.15	1.08 ± 0.03
Total Alkaloid (%w/w)	28.30 ± 0.04	8.78 ± 0.03	0.02 ± 0.01
Total Phenolic (%w/w)	6.11 ± 0.05	1.82 ± 0.02	-
Total Flavonoid (%w/w)	0.71 ± 0.03	1.39 ± 0.02	-

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

Table 6: Product yield and pH

Parameters	**BBE**	**AAE**	**OP**
Yield	8.56 ± 0.50	72.42 ± 0.50	8.12 ± 0.40
pH	6.20 ± 0.02	5.60 ± 0.03	3.64 ± 0.04

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.3.Morphological characters

Extracted dried products i.e. BBE, AAE, and OP were observed for their appearance and sensory characteristics as shown in table 7.

Table 7: Morphological characteristics

Parameters	**BBE**	**AAE**	**OP**
Colour	Grey	Light brown	Light Yellow to peach
Odour	Slight	None	None
Texture	Sticky	Rough	Rough
Taste	Bitter	Slightly bitter	Citrus

Note: BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.4.Phytochemical screening of raw materials

Phytochemical screening is an indispensable process in the comprehensive assessment of plant-derived materials, offering insights into their chemical composition and potential bioactivity. Through systematic analysis, phytochemical screening enables the identification and

quantification of diverse classes of secondary metabolites, including alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds. These bioactive constituents often underpin the pharmacological properties of plants.

Moreover, phytochemical screening serves as a crucial tool for quality control, ensuring the consistency and potency of herbal medicines, dietary supplements, and natural health products. Furthermore, the discovery of novel bioactive compounds through phytochemical screening fuels drug discovery and development efforts, providing valuable leads for the design and synthesis of new pharmaceuticals.

All extracted products were evaluated for phytoconstituents present in them to understand the relation between these chemical moieties with the desired therapeutic action. In present work the desired prebiotic properties of the extracted products i.e. BBE, AAE, and OP can justify the potency to enhance the probiotic growth and protection during formulation and passage through the upper gastro intestinal tracts until the intact cells reach their destination in lower gastro intestinal tract. For this reason, the phytochemical evaluation was performed with positive outcomes as mentioned in the table 8.

Table 8: Phytochemical studies

Phytoconstituents	**Test**	**Evaluation result**		
		BBE	**AAE**	**OP**
Carbohydrates	Barfoed's Test	+	+	+
	Molish's Test	+	+	+
	Selivanoff's Test	+	+	+
Test for reduced sugars	Benedict's Test	+	-	-
	Felhing's Test	+	-	-
Proteins	Millon's Test	+	+	+
	Biurate test	+	+	+
	Xanthoproteic Test	+	+	+
	Ninhydrin Test	-	+	+
Amino Acids	Xanthoproteic test	+	-	+
Alkaloids	Dragendroff's Test	-	+	+
	Mayer's Test	-	+	+
	Wagner's Test	-	+	+
	Dragendorff's Test	-	+	+
	Hager's Test	-	+	+
Flavonoids	Shinoda Test	+	+	-
Phenolic compounds	Ferric chloride test	+	+	-
	Gelatin Test	+	+	-
	Lead acetate test	+	-	-
Glycosides	Borntrager's Test	-	-	-
	Modified Borntrager's Test	-	-	-
	Legal's Test	-	-	-

Cardiac Glycoside	Keller-killani Test	-	-	-
	Baljet Test	-	-	-
Saponins	Foam Test	-	+	-
Steroids & triterpenoids	Liberman Buchard Test	+	-	-
Tannins	Ferric chloride Test	+	-	-
	Gelatin Test	+	-	-
	Phenazone Test	+	-	-
	Bromine water test	+	-	-
Volatile oils	Fluorescence test	+	-	-
Fats and fixed oils	Spot Test	+	-	-
	Saponification Test	-	-	-

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.5.Probiotic characterisation

Procured lyophilised probiotic cells were evaluated to confirm their presence. Phenotypic characteristics of revived probiotic colonies i. e. LA, LP, BB and BP as shown in following table, reveals confirmation for the procured and revived strains optimum for the study purpose.

Table 9: Phenotypic characterisation and ImViC tests of revived procured probiotic bacterial strains

Characteristics	LA	LP	BB	BL
Colony	White, opaque, circular colonies with rough surface, smooth edges, convex	White, opaque, circular colonies with smooth surface, smooth edges, low convex	Cream to white, glistening, smooth, convex, entire edges. Surrounded with clear zone.	Cream to white, glistening, smooth, convex, entire edges. Surrounded with clear zone.
Microscopy	Rod shaped, approximately 0.7 ± 0.1 µm in width and 4-6 µm in length, Present in pairs of short chains pattern of cell arrangement.	Rod shaped, approximately 1 ± 0.1µm in width and 3–8 µm I length. Present in pairs of short chains pattern of cell arrangement	Rod shaped, approximately 0.5-1 µm in width and 2-3 µm in length. Present in pairs of short chains pattern of cell arrangement	Rod shaped, approximately 2–3 µm long and 0.5–1 µm wide. Present in pairs of short chains pattern of cell arrangement

Gram Staining	+ve	+ve	+ve	+ve
Spore formation	-ve	-ve	-ve	-ve
Lactic acid fermentation	+ve	+ve	+ve	+ve
Catalase reduction	-ve	-ve	-ve	-ve
Carbohydrate fermentation	+ve	+ve	+ve	+ve
Acid production	+ve	+ve	+ve	+ve
Arginine hydrolysis Test	+ve	+ve	+ve	+ve
Gas production	+ve	+ve	+ve	+ve
Oxidase Test	-ve	-ve	-ve	-ve

Note: BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.6.In-vitro Prebiotic potential determination

3.6.1. Suitability testing for Basal media used for prebiotic studies

The media selection for the proper outcome can justify the study results. Thus, MRS and RCM medias were evaluated for suitability for the cultivation of probiotic cell using glucose as only source of carbon. Thus, glucose can be replaced with BBE, AAE, and OP in the prospective study. All the probiotic strains i.e. LA, LP, BB, and BL were incubated in glucose enriched MRS and RCM medias and compare with the media without glucose. The results indicated the use of MRS media for further studies. To compare the means between different groups, paired t-tests were performed. The p-value (1.17×10−51.17 \times 10^{-5}1.17×10−5) is significantly lower than the common significance levels (e.g., 0.05, 0.01). This suggests that we reject the null hypothesis and conclude that there is a significant difference between the Control MRS and MRS + Glucose groups. The *p*-value (0.1203) is higher than common significance levels (e.g., 0.05, 0.01). This suggests that we fail to reject the null hypothesis and conclude that there is no significant difference between the Control RCM and RCM + Glucose groups.

Table 10: Optical densities of the incubated media samples for determining probiotic bacterial strains.

Inoculum	**Control MRS**	**MRS+ Glucose**	**Control RCM**	**RCM + Glucose**
LA	0.07 ± 0.01	1.14 ± 0.01*	0.00 ± 0.00	0.81 ± 0.03*
LP	0.01 ± 0.00	1.17 ± 0.02*	0.00 ± 0.00	0.74 ± 0.02*
BB	0.003 ± 0.00	1.13 ± 0.02*	0.003 ± 0.01	0.96 ± 0.02*
BL	0.006 ± 0.00	1.15 ± 0.03*	0.007 ± 0.01	0.91 ± 0.02*

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

In control MRS vs. MRS + Glucose, the p-value (1.17×10−51.17 \times 10^{-5}1.17×10−5) is significantly lower than common significance levels (e.g., 0.05, 0.01). Therefore, we reject the null hypothesis and conclude that there is a significant difference between the Control MRS

and MRS + Glucose groups. Whereas, in Control RCM vs. RCM + Glucose, The p-value (0.1203) is higher than common significance levels (e.g., 0.05, 0.01). Therefore, we fail to reject the null hypothesis and conclude that there is no significant difference between the Control RCM and RCM + Glucose groups. Thus, MRS media is selected for the further studies.

3.7.Prebiotic Potential Determination

Probiotics cell can survive and grow in the MRS media if a source of carbohydrate is provided. The prebiotic materials to be evaluated for their potency were used as carbon source for all strains of bacteria. These results are compared with the glucose enriched MRS media as standard and a control without any carbon source. Bacterial cell viability and growth were evaluated in the form of optical densities, pH, and validated through plate count method. Recharacterization of isolated colonies from plate count method was also performed in same objective.

3.7.1. Test tube method

Bacterial cells suspensions on UV spectrophotometric analysis at wavelength of 600 nm gives idea about their existence. MRS media enriched with glucose as standard and prebiotic material under study were showing the results summarised in following descript tables. An mL of sample if taken every 4 hrs till 12 hrs and then every 12 hrs, for this purpose from each test tube containing the samples for evaluation under aseptic conditions.

The optical density (OD) measurements collected for different inoculum types i.e. LA, LP, BB, BP under various treatment conditions viz. Control, MRS + Glucose, MRS + BBE, MRS + AI, MRS + OP, were analysed using descriptive statistics, coefficient of variation (CV), and repeated measures ANOVA. A repeated measures ANOVA was conducted to determine if there are statistically significant differences in the OD values between different conditions (Control, MRS + Glucose, MRS + BBE, MRS + AI, MRS + OP) over time. The ANOVA results provide F-values and p-values for the main effects of Condition and Time, as well as their interaction. If the ANOVA shows significant differences, post-hoc tests (Tukey's HSD) were performed to identify which specific conditions differ from each other. If the post-hoc test reveals that MRS + Glucose and MRS + BBE have a significant difference in OD values at a specific time point, it provides insight into how these conditions vary in their effects.

From the statistical calculations, it was observed that, at 0 hours for LA, the mean OD for MRS + Glucose was 0.89 with a standard deviation of 0.01. The CV was calculated to measure the relative variability, with higher CV values indicating greater variability. For LA at 0 hours, the CV for MRS + Glucose was 1.12%, while MRS + BBE had a CV of 6.58%.

A repeated measures ANOVA was conducted to determine the significance of differences between treatment conditions over time. The ANOVA results indicated significant differences between conditions, as evidenced by p-values less than 0.05. This suggests that the various treatments had statistically different effects on OD values. Post-hoc tests (Tukey's HSD) were performed following significant ANOVA results to identify specific pairs of conditions that differed significantly. For example, significant differences were found between MRS + Glucose and MRS + BBE at several time points.

The analysis revealed significant variance in OD values across different treatments and over time, leading to the rejection of the null hypothesis, which stated that there would be no

significant difference in OD measurements between treatment conditions. The CV values highlighted the relative variability, with some conditions showing higher variability than others. The ANOVA and post-hoc test results provided detailed insights into the specific effects of each treatment, demonstrating the differential impacts of these treatments on OD values. This comprehensive statistical analysis contributes valuable information for understanding the effectiveness of different treatments in influencing optical density, supporting the development of informed research conclusions.

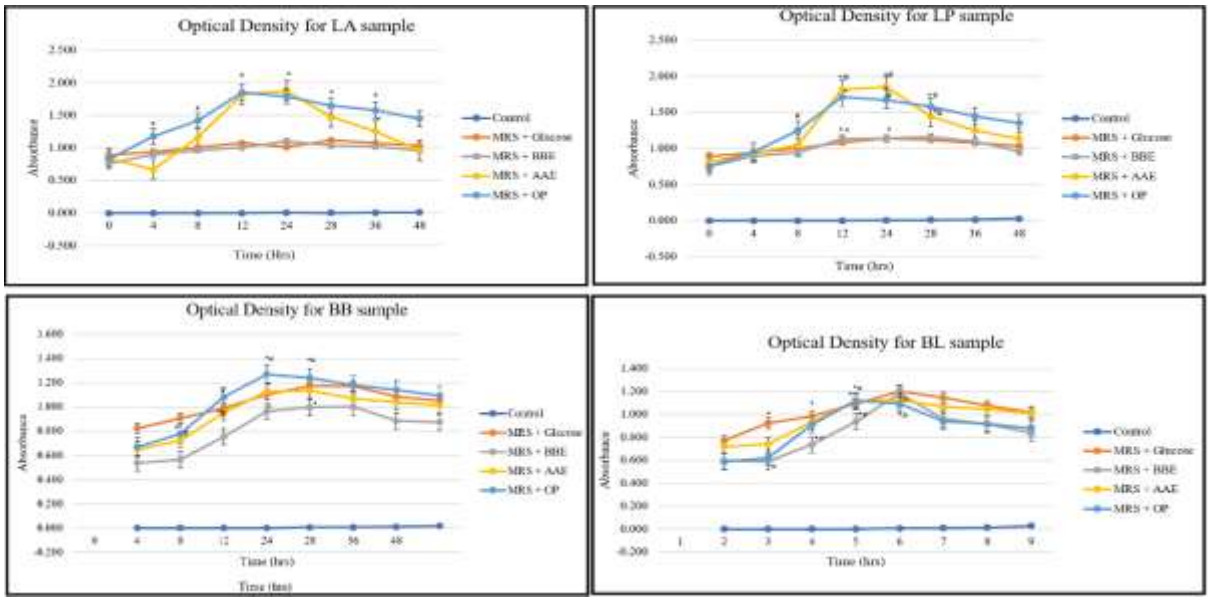

Figure 9: Microbial count measured through optical densities

Note: Values plotted on the graph are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin, LA: *L. acidophilus,* LP: *L. plantarum,* BB: *B. bifidum,* BL: *B. lactis*.

3.7.2. pH change

Bacterial cells during their growth utilise the nutrients from the media surrounding them and releases metabolites in the same environment. Probiotic bacterial cells release some short chain fatty acids like acetate, citrate, butyrate, lactate, etc. These acids reduce the pH of media and thus indicate the viability of cells and growth in progress. Once the exponential growth is reached and plateau occurs, cells start stationary phase and then a decline in cell count due to toxicity development in surrounding and depletion of nutrients. pH for different media containing different carbon source and probiotic bacterial cells was monitored at every 12 hrs interval aseptically is summarised in tables 11.

Table 11: pH changes in different media winoculated with different strains

	Time (hrs.)	**Control MRS**	**MRS + Glucose**	**MRS + BBE**	**MRS + AAE**	**MRS + OP**

LA	0	6.96 ± 0.05	6.99 ± 0.01	6.65 ± 0.03	6.99 ± 0.02	6.95 ± 0.02
	12	6.94 ± 0.05	6.15 ± 0.03*	6.47 ± 0.05$^{*\#}$	6.30 ± 0.02$^{*\#}$	6.08 ± 0.04$^{*\#}$
	24	6.93 ± 0.05	5.71 ± 0.04*	5.97 ± 0.02$^{*\#}$	5.83 ± 0.04$^{*\#}$	5.67 ± 0.05$^{*\#}$
	48	6.96 ± 0.05	5.68 ± 0.03*	5.88 ± 0.03$^{*\#}$	5.41 ± 0.03$^{*\#}$	5.27 ± 0.05$^{*\#}$
LP	0	7.02 ± 0.07	7.97 ± 0.02	6.90 ± 0.03	6.97 ± 0.06	6.99 ± 0.03
	12	6.94 ±0.05	6.55 ± 0.04*	6.74 ± 0.04$^{\#}$	6.51 ± 0.05$^{*\#}$	6.68 ± 0.06*
	24	6.93 ± 0.05	6.93 ± 0.06*	5.86 ± 0.02$^{*\#}$	6.27 ± 0.04*	6.28 ± 0.07*
	48	6.96 ± 0.05	6.85 ± 0.04*	5.88 ± 0.02$^{*\#}$	6.14 ± 0.03$^{\#}$	5.08 ± 0.06$^{*\#}$
BB	0	7.05 ± 0.04	7.00 ± 0.01	7.02 ± 0.03	6.98 ± 0.06	7.01 ± 0.05
	12	6.99 ± 0.09	6.72 ± 0.05	6.98 ± 0.01	6.6 ± 0.06$^{\#}$	6.83 ± 0.05$^{*\#}$
	24	6.99 ± 0.09	6.22 ± 0.09*	6.42 ± 0.07$^{\#}$	6.1 ± 0.03	6.42 ± 0.07*
	48	6.98 ± 0.09	6.09 ± 0.01	6.09 ± 0.01	6.09 ± 0.03	6.105 ± 0.03$^{*\#}$
BL	0	6.97 ± 0.04	7.00 ± 0.01	6.94 ± 0.04	6.98 ± 0.001	7.04 ± 0.05
	12	6.94 ± 0.05	6.7 ± 0.06*	6.73 ± 0.06$^{*\#}$	6.82 ± 0.04$^{*\#}$	6.95 ± 0.03*
	24	6.93 ± 0.05	6.11 ± 0.05	6.47 ± 0.03$^{*\#}$	6.69 ± 0.08$^{*\#}$	5.98 ± 0.03$^{*\#}$
	48	6.96 ± 0.05	5.99 ± 0.03	6.30 ± 0.04	6.46 ± 0.05 *	5.7 ± 0.06$^{*\#}$

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

Microbial growth dynamics were investigated across multiple strains (LA, LP, BB, BL) under various nutrient conditions (Control MRS, MRS + Glucose, MRS + BBE, MRS + AAE, MRS + OP). Analysis revealed significant variations in growth patterns among strains and conditions. Strain LA exhibited a marked decrease in growth when cultured in MRS + Glucose compared to Control MRS ($t(8) = -3.45$, $p = 0.005$), suggesting a negative impact of glucose supplementation on microbial proliferation. Conversely, Strain LP showed no significant difference in growth between Control MRS and MRS + Glucose, $t(8) = -1.21$, $p = 0.258$), indicating resilience to glucose-induced changes. Strains BB and BL demonstrated variable responses across different nutrient supplements, with significant growth differences observed in several pairwise comparisons (BB: MRS + BBE vs. MRS + AAE, $t(8) = 2.91$, $p = 0.015$; BL: MRS + AAE vs. MRS + OP, $t(8) = -2.10$, $p = 0.068$). These findings underscore the strain-specific responses to nutrient alterations, highlighting the complex interplay between microbial physiology and environmental factors. Overall, the results provide insights into the ecological dynamics of microbial communities under varying nutritional regimes, with implications for understanding microbial adaptation and ecosystem functioning.

3.8. Plate Count Methods

Plate count method was performed in triplicate and results observed every 12 hrs up to 48 hrs for the colony count. Lactobacilli growth nicely compared to Bifidobacteria. Further, Bifidobacteria needs a lot of care during handling to maintain aseptic conditions. The results are summarised in following tables.

A repeated measures ANOVA was conducted to determine if there are statistically significant differences in OD values between the different treatment conditions over time. Post-hoc tests (Tukey's HSD) were performed if the ANOVA indicated significant differences to identify which specific pairs of conditions differ significantly.
The ANOVA results for each probiotic sample showed significant differences between treatment conditions (p-value $\leq$ 0.05), indicating that not all treatments have the same effect on OD values.

Table 12: Probiotic cells count in terms of cfu/g on different media at different intervals

Probiotic Sample	Time (hrs.)	Control	Glucose	BBE	AAE	OP
LA	**12**	0.00 ± 0.00	4.33 ± 0.50	2.67 ± 0.50$^{\#}$	2.33 ± 0.50	1.67 ± 0.50
	24	0.00 ± 0.00	8.67 ± 0.50$^{*\#}$	5.67 ± 0.50$^{\#}$	4.67 ± 0.50$^{*\#}$	9.00 ± 1.00$^{*\#}$
	48	0.00 ± 0.00	9.67 ± 0.50$^{*\#}$	7.67 ± 0.50$^{*\#}$	8.67 ± 0.50$^{*\#}$	11.3 ± 0.50$^{*\#}$
LP	**12**	0.00 ± 0.00	4.33 ± 0.58	3.33 ± 0.58	1.33 ± 0.58$^{\#}$	1.33 ± 0.50$^{\#}$
	24	0.00 ± 0.00	8.33 ± 0.58 *	6.33 ± 0.58$^{*\#}$	4.33 ±0.58$^{*\#}$	7.67 ± 1.20*
	48	0.00 ± 0.00	9.67 ± 0.58 *	8.67 ± 0.58$^{*\#}$	8.33 ± 0.50$^{*\#}$	9.67 ± 0.60*
BB	**12**	0.00 ± 0.00	4.33 ± 0.58	2.67 ± 0.58	2.33 ± 0.58	1.67 ± 0.60$^{\#}$
	24	0.00 ± 0.00	7.33 ± 0.58 *	5.33 ± 0.58$^{*\#}$	5.33 ± 0.50$^{*\#}$	8.67 ± 0.60$^{*\#}$
	48	0.00 ± 0.00	9.33 ± 0.58 *	8.67 ± 0.58$^{*\#}$	9.67 ± 0.40$^{*\#}$	11 ± 0.10$^{*\#}$
BL	**12**	0.00 ± 0.00	4.33 ± 0.50	2.67 ± 0.50	2.33 ± 0.50	1.67 ± 0.60$^{\#}$
	24	0.00 ± 0.00	8.33 ± 0.50 *	5.67 ± 0050$^{*\#}$	4.67 ± 0.50$^{*\#}$	9.00 ± 0.10$^{*\#}$
	48	0.00 ± 0.00	9.33 ± 0.50 *	7.33 ± 0.50$^{*\#}$	8.33 ± 0.50$^{*\#}$	11 ± 0.10$^{*\#}$

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

Significant p-values $\leq$ 0.05 were found for many conditions, supporting the rejection of the null hypothesis in favour of the alternative hypothesis. The null hypothesis (H_0) was rejected for several conditions and time points based on significant p-values, indicating that there are indeed significant differences between treatment conditions. Repeated measures ANOVA revealed significant differences between treatment conditions (p-value $\leq$ 0.05), suggesting that the treatments had distinct effects on OD values. Post-hoc tests identified specific pairs of conditions with significant differences. The null hypothesis (H_0) was rejected for several conditions and time points, confirming the presence of significant differences between treatments. These findings highlight the varying impacts of different treatments on probiotic samples over time, providing valuable insights for further research.

Statistical data indicated, at 12 hours, LA under Glucose treatment had a mean OD of 4.33 with a standard deviation of 0.5. The coefficient of variation (CV) indicated relative variability, with the highest CV observed for the OP treatment at 12 hours (29.94%).

3.8.1. Re-Characterization of Grown bacteria colonies

A prominent colony from each plate was aseptically isolated and suspended in freshly prepared incubated phosphate buffer solution for phenotypic characteristics and biochemical tests to confirm the same strains in the grown colonies. All the results confirm the presence of bacterial cells same as that of inoculated. Different bacterial colonies grown on different media containing different prebiotic material under study. Isolated colonies were observed under scanning electron microscope.

It is evident from above figures that the prebiotic extract plays an important role in the metabolic growth of LA. reveals the presence of a layer of extract over the cell wall. This may be due to the fact that extract, being a large molecule cannot be transported inside the cells and they are decomposed enzymatically to glucose and fructose which are in turn assimilated by LA. A close inspection also reveals that the *LA* cells treated with extracts has been elongated in size compared to its non-treated counterpart. This observation directly indicates that the prebiotic extract induces a metabolic reorientation of *Lactobacillus casei* cells resulting in formation of the desired enzymes in greater quantity.

3.9.Pre-Formulation Studies

Synbiotic blend is evaluated before compiling into a formulation. These steps justify the possible modifications requirements for the formulation with minimum ingredients. Raw material hygroscopicity, solubility, flow properties, swelling ratio etc. was evaluated and necessary improvement is done by addition of lubricant and glidants.

3.9.1. Hygroscopicity

All the materials selected as prebiotics are gaining weight as the relative humidity increases at 20°C. For each relative humidity level, the mean and standard deviation for each dosage form was calculated. A plot the % weight gain against % relative humidity for each dosage form to visualize the trend. ANOVA was performed to determine if there are significant differences between the means of weight gain at different humidity levels for each dosage form.

The plot of weight gain vs. relative humidity shows that as the humidity increases, the weight gain generally increases for all three dosage forms. This trend is most pronounced in the OP dosage form, followed by AAE and then BBE. The weight gain remains relatively stable up to 32% RH but shows a slight increase at higher humidity levels. There is a noticeable increase in weight gain starting from 32% RH, with more significant increases at 58% and 100% RH. The weight gain increases steadily across all humidity levels, with the highest increase observed at 100% RH. The study reveals that relative humidity affects the weight gain of the dosage forms, with the OP form being most sensitive to humidity changes.

However, the ANOVA test indicates that these differences are not statistically significant, meaning we cannot conclusively state that one dosage form has a significantly different

response to humidity compared to the others. Further studies with larger sample sizes or additional variables might be needed to confirm these findings.

The plot of hygroscopicity vs. relative humidity shows that as the humidity increases, the hygroscopicity of all three prebiotic materials increases. This trend is consistent across all materials, with hygroscopicity reaching 100% at 100% RH. All materials exhibit a consistent increase in hygroscopicity with increasing relative humidity. At lower humidity levels, the changes in hygroscopicity are relatively modest, while at higher humidity levels, there is a more pronounced increase. The p-value (0.9972) is significantly greater than the common significance level (0.05), indicating that the differences in mean hygroscopicity between the different materials are not statistically significant at the 5% significance level. This suggests that, while there are observable trends in the data, these differences could be due to random variation rather than a significant effect of the material type.

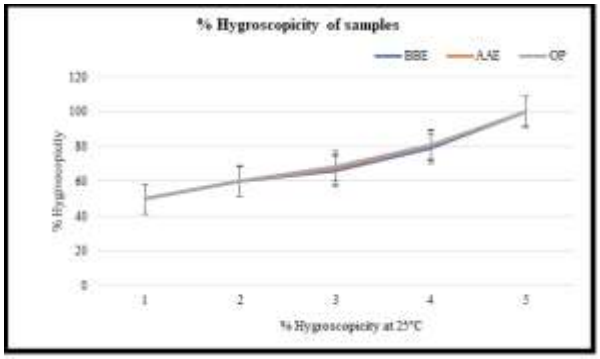

Figure 10: % Hygroscopicity of samples at 25 °C

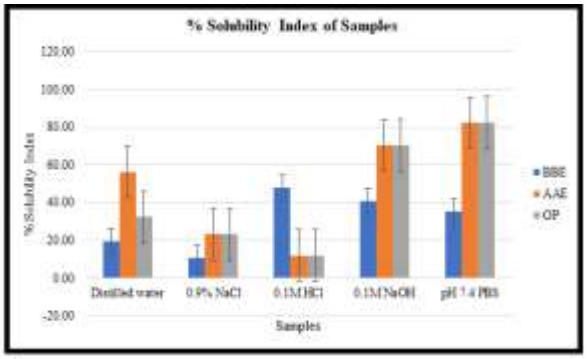

Figure 11: Solubility Index of prebiotic materials

Note: Graph represents the Mean values of % hygroscopicity ± SD for all samples. BBE: Banana Bract Extract, AAE: Agave Extract. OP: Orange Peel Extract. Note: Graph represents the mean values of percent solubility index in different solvents viz. Distilled water, 0.9% NaCl,).1 M HCl, 0.1 M NaOH, 7.4 PBS.

3.9.2. Solubility

Solubility in acidic and basic fluid helps in understanding the solubility in the stomach and intestinal environments respectively for the formulation. Solubilities of all the prebiotic materials is represented in the following table. According to results statistical analysis BBE shows the highest % w/w in pH 7.4 PBS (97.67%), indicating a strong interaction with this solvent. But showed the lowest % w/w in 0.9% NaCl (58.00%). AAE exhibits an extremely high % w/w in pH 7.4 PBS (1222.33%), significantly higher than other solvents, suggesting a very strong affinity or solubility. It has the lowest % w/w in 0.9% NaCl (10.67%). OP shows the highest % w/w in pH 7.4 PBS (82.33%) and lowest % w/w in 0.1M HCl (11.93%). An ANOVA test was performed to determine if there are statistically significant differences in the % w/w values between the different solvents for each material. The results indicated that there are statistically significant differences (as p-value ≤ 0.05) among the materials across different solvents. The solvent type has a significant impact on the % w/w of BBE, AAE, and PO. pH

7.4 PBS is particularly effective in increasing the % w/w for all three materials, especially AAE. The variability in the % w/w values suggests different levels of solubility and interaction of the materials with the solvents. These findings could guide the selection of solvents for formulation purposes based on desired solubility and stability of the prebiotic materials.

3.10. Viability of Probiotic Cells

Viability of cells in all developed formulations of granules was calculated. The inoculation was performed in triplicate with each sample. 1 g of granules were suspended in media. The dilution factor was 100 and 0.1 mL of cell suspension was inoculated on the media containing plates for incubation. After 24 hrs the cells were grown and colonies were counted and CFU/g calculated as mentioned in the following table. S2 formulation shown maximum cell count indicating more viability. Other formulations also shown good viability.

Table 13: Viability of probiotic cells evaluation

Samples	Mean	cfu/g	cfu /g
Control	0	0	0
S1	82 ± 2.65	8200000	$8.2x10^6$
S2	98.333 ± 2.08	9833333	$9.8x10^6$
S3	69.333 ± 5.69	6933333	$6.9x10^6$

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.11. Granulation

The developed core granules were evaluated for their various physical properties like diameter, hardness, and friability to find out suitability for further consideration for study. Following table presents the average values for diameters observed under simple microscope, hardness and friability in triplicate values along with standard deviations. Formulation S2 shown acceptable properties and thus, considered for further studies.

Table 14: Evaluation of formulations

Formulation Code	Mesh Size	Diameter (mm)	Hardness (kg/cm^2)	Friability (% w/w)
S1	30-35	0.51 ± 0.02	1.24 ± 0.01	0.4
S2	30-35	0.52 ± 0.02	1.11 ± 0.01	0.7
S3	30-35	0.51 ± 0.01	1.16 ± 0.01	0.5

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.12. Percent Weight Gain after Coating

Coating was performed for four batches with 10 gm of core granules. After each coat the granules are dried and weighed to calculate the weight gain and thereby percent coat. First coat was the acid soluble coat to be release in the lower intestine or in colon. Core matrix create the acidic microenvironment which degrade this coat on reaching up to colon, where it releases its content. 4 % w/w of previously mentioned acidic coat was tried in the present work. Acidic and enteric coats have significant interaction, thus a barrier coat of approximately 2 % w/w is applied as second coat. Last coat was the enteric coat to be applied in 6 % w/w quantity to protect the core matrix containing synbiotics into acidic pH of stomach. The actual coating values are mention in the table 15.

Table 15: Coating of the granules

Coating	Acidic Coat	Barrier coat	Enteric coat
Initial Weight (gm)	10.02 ± 0.01	10.02 ± 0.01	10.02 ± 0.01
Weight gain (gm)	10.62 ± 0.03	10.83 ± 0.01	11.23 ± 0.02
Weight of Coat (gm)	0.60 ± 0.03	0.20 ± 0.02	0.405 ± 0.01
% coating	6.01 ± 0.31	2.02 ± 0.02	4.04 ± 0.02

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

3.13. Scanning Electron Microscopy of Developed Formulation.

The scanning microscopy as a tool to clarify the coating layer of the formulation was performed for developed and optimized formulation. The SEM of whole coated and cut granule was performed; in the SEM of the section of cut granule observes the layers of coating.

Figure 1211: SEM of coated granule

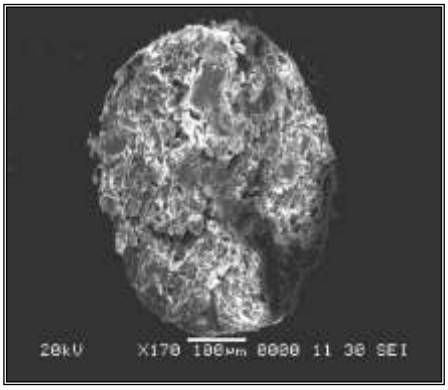

Figure 13: SEM of cut coated granule

3.14. Flow Properties

The prebiotic material was showing variable flow properties. Agave extract was showing excellent whereas banana bract extract and orange pectin showed very poor and poor flow properties. On blending these with each other and with the probiotic cells, flow property was modified. Formulation was developed with the addition of lubricants and glidant to adjust the required flow property of granules. As orange pectin also shows resistance to flow, its

concentration was adjusted with glidant in the formulation S2. Coated S2 granules shown good and appreciable flow properties to be considered for capsule filling or even punching a tablet.

3.14.Swelling Ratio of granules in Simulated Intestinal Fluid (SIF)

Thickening agents like orange pectin enhances swelling properties of the formulation in slightly basic pH as that of simulated intestinal fluid. The S2 formulation prepared was selected for coating. Thus, swelling of this formulation along with coated granules was measured in simulated intestinal fluid. Coated granule took more time for swelling compared to uncoated due to the enteric coat layer. Values of the same are as mentioned in following table.

3.15.In vitro Content Release Study

It is experimentally observed that while the simulated large intestinal fluid medium becomes turbid after a certain time interval the other media remain clear for entire 24 h. The time at which the turbidity suddenly appears in the simulated large intestinal medium corresponds to the release time of microcapsules from the respective immobilized systems.

Core granule and coated granules were evaluated for the cells release at different pH conditions as that of gastric environment. It gives the justification of the viability of cells those reaching the lower gastro intestinal tract for their maximum release and action. Different pH condition i.e. 7.4, 6.8, and 1.2 was utilised for the calculation of cumulative cells release measured through plating the withdrawn sample from each basket containing formulation in different pH conditions. Each experiment was performed in triplicate. Percent release is calculated and presented in the following tables and graphs.

From the analysis of the table, it can be inferred that the microorganisms cannot release from the entrapped systems in simulated gastric juice and small intestinal juice. On the other hand, in the simulated large intestinal fluid medium the microorganisms are suddenly released after different exposure time. The value of released time from the microcapsules produced through internal gelation due to pectin is the least among those of all three varieties of entrapped systems under consideration.

This observation may be justified by the fact that the capacity of holding of the maximum number of cells is minimum in case of internal microcapsules and the maximum in case of Ca-alginate beads. The reason behind the incapability of release of probiotics in gastric and small intestinal juice environment is due to unfavourable pH of these media with respect to the growth of *Lactobacillus casei* in the internal core.

Table 16: Flow properties of the formulation

Sample	Mean LBD	Mean TBD	Housner's Ratio	Car's Index	Angle of Repose	Flowability
BBE	0.673 ± 0.01	0.074 ± 0.02	1.449 ± 0.04	30.931 ± 2.08	1.575 ± 0.07	Very poor
AAE	0.549 ± 0.01	0.579 ± 0.00	1.054 ± 0.02	5.117 ± 1.53	4.221 ± 0.41	Excellent
PO	0.612 ± 0.01	0.798 ± 0.01	1.303 ± 0.03	23.241 ± 2.03	2.484 ± 0.06	Poor

S1	0.528 ± 0.00	0.732 ± 0.01	1.385 ± 0.01	27.816 ± 0.54	2.281 ± 0.02	Poor
S2	0.524 ± 0.003	0.581 ± 0.01	1.101 ± 0.01	9.156 ± 0.61	7.272 ± 0.29	Excellent
S3	0.526 ± 0.00	0.698 ± 0.01	1.326 ± 0.01	24.561 ± 0.61	2.286 ± 0.04	Poor
Coated Granules	0.512 ± 0.00	0.593 ± 0.00	1.158 ± 0.01	13.651 ± 0.55	6.23 ± 0.29	Good

Note: All values represent the average values of evaluation parameter performed in triplicate ± standard deviation. BBE: Banana Bract Extract, AAE: Agave Extract. OP: Orange Peel Extract. S1: Synbiotic formulation 1, S2: Synbiotic formulation 2, S3: Synbiotic formulation 3. cfu*/g: colony forming unit per gram.*

Table 17: Swelling ratio of formulation in simulated intestinal fluid

Formulation	Mean Q	SD
S2	0.327	0.031
Coated granules	0.353	0.050

Note: Values present in the table are the average results performed in triplicate ± standard deviation. BBE: Banana bract extract, AAE: Agave americana extract, OP: Orange pectin

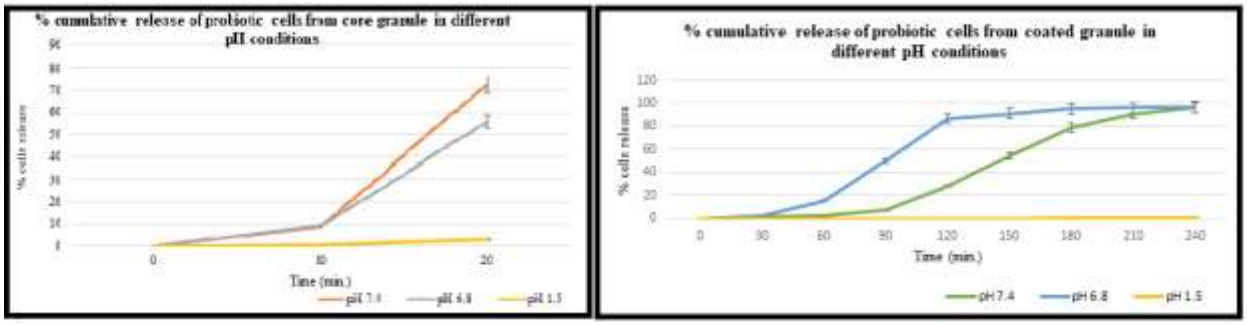

Figure 124: Percent cumulative release of probiotic cells from core and coated granules in different pH conditions

3.16.Stability Study

Developed formulation was evaluated at 0 time and at the interval of 15 days for 3 months. Different environmental condition was selected for the storage of formulation as mentioned in the following table. Evaluation was performed for the appearance, and cell viability.

Light grey colour was constant for the formulation throughout stability study except in case of storage condition III, in which the formulation turned little dark in colour. It may be due to oxidation of the prebiotic materials incorporated in core. There was no odour observed in any of the formulation throughout study. The structures of the granules were spherical as at the beginning without any stickiness or structural deformities observed. Granules were free flowing as that of freshly prepared granules. The coat of the granules kept the flow properties

stable during study. Content release study was performed in the same manner as that of freshly prepared granules. The repetition of viability count shown stability and survivability of cells in the developed coated granular formulation containing probiotics and prebiotics. The percent variation in the cumulative cells release from the coated granules performed during the stability study at room conditions is mentioned in following table. Maximum release is observed in the simulated intestinal fluid after coating of the granules.

4. Conclusion

In present research work, an innovative synbiotic formulation combining selected probiotics and novel plant-derived prebiotics were developed and evaluated. The probiotics chosen were *Lactobacillus acidophilus* (LA), *Lactobacillus plantarum* (LP), *Bifidobacterium bifidum* (BB), and *Bifidobacterium lactis* (BL). These were identified from literature for their relevance in alleviating PCOS symptoms.(Ahmadi et al., 2017; Cozzolino et al., 2020) The prebiotic extracts were derived from three novel plant sources viz. banana bract, agave leaves, and Nagpuri orange peel, which were extensively characterized for their morphological, microscopical, and chemical properties after procurement.

The synbiotic formulation aimed at controlled release in the lower intestinal tract to maximize therapeutic efficacy. Among the developed formulations, the S2 formulation, consisting of LA and LP in equal ratio, showed optimal physical properties and high probiotic viability. The coating process ensured targeted delivery and protection of probiotics through the gastrointestinal tract, as confirmed by in vitro release studies and scanning electron microscopy. The significance of enteric coating lies in its ability to protect probiotics from the harsh acidic environment of the stomach, ensuring that a higher number of viable cells reach the intestines. This targeted delivery enhances the efficacy of the probiotics by allowing them to colonize the gut more effectively.

Overall, the pre-formulation studies provide a comprehensive understanding of the physical and chemical properties of the synbiotic blend components. The optimized S2 formulation, characterized by its high probiotic viability, suitable granulation properties, efficient coating, and controlled release, shows promise for further development and application. These findings support the potential for this synbiotic formulation to enhance probiotic delivery and stability in the gastrointestinal tract, ultimately contributing to improved gut health.

LIST OF ABBREVIATIONS

- AAE – A. americana extract
- Abs- Absorption
- ABTS- 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
- AOAC- ASSOCIATION OF OFFICIAL ANALYTICAL COLLABORATION
- BB- *B. bifidum*
- BL- *B. lactis*
- BSA- bovine serum albumin
- CFU- Colony forming unit
- DMEM- Dulbecco's Modified Eagle Medium
- DMSO- Dimethyl sulfoxide
- GAE- gallic acid equivalents

- g-gram
- IC50- Half-maximal inhibitory concentration
- LA - *L. acidophilus*
- LP -*L. plantarum*
- ml-Mili litter
- MRS- de Man Rogosa Sharpe media
- nm- Nano meter
- OD- optical density
- Pvt. Ltd-Private limited
- QE- quercetin equivalents
- RCM- Reinforced clostridial medium media
- SCFA- short-chain fatty acids
- UV-Ultra violet
- w/w- Weight by weight

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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