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A comprehensive investigation into prebiotic influence of orange pectin on synergistic modulation of probiotic viability

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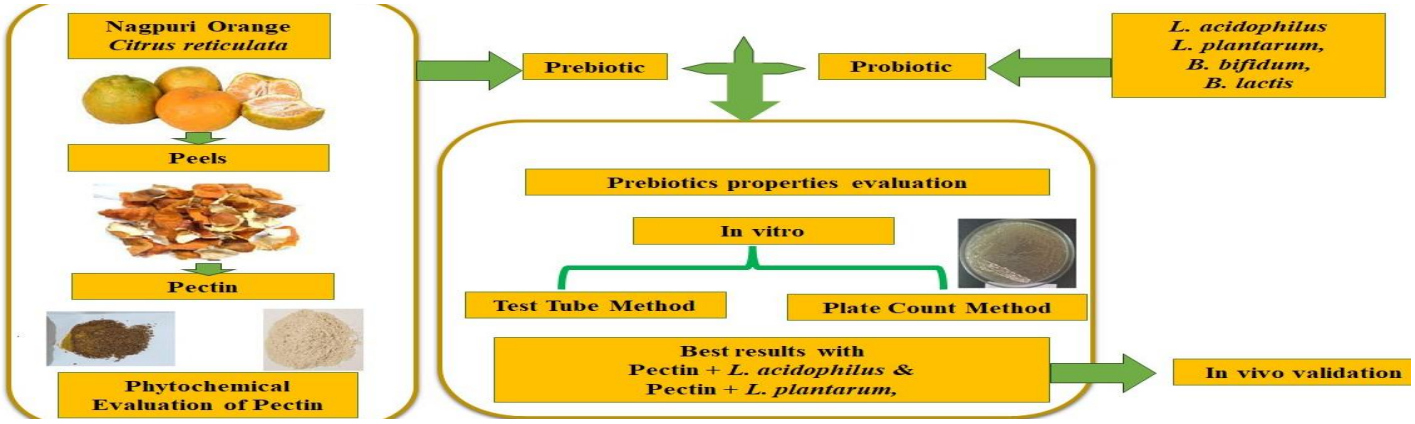
ABSTRACT

Synbiotics are well accepted for their nutraceutical value and also in therapeutics for lifestyle disorders like diabetes, cardiovascular, neurological diseases and PCOS. Current manuscript presents the study of prebiotic potential of Pectin isolated from Nagpuri orange on the strains of probiotic bacteria like L. acidophilus, L. plantarum, B. bifidum, and B. lactis. In vitro and in vivo evaluation shows a significant increase in the L. acidophilus colonies as 2.12 ± 0.04 cfu/g followed by L. plantarum as 1.38 ± 0.03 cfu/g, B. lactis as 0.68 ± 0.04 cfu/g and B. bifidum as 0.48 ± 0.05 cfu/g using a modified MRS media. The highest cfu/g complemented the declined pH of cultured media to 6.05 ± 1.32 from near neutral pH. In vivo study using Sprague Dawley rats presented enhanced fecal colony count for L. acidophilus as 8.92 ± 0.04 cfu/g along with confirmation for microscopical characteristics of Gram-positive, rod-shaped, non-motile, non-sporulating bacteria showing catalase-negative, carbohydrate fermentation, lactic acid production, and positive arginine hydrolysis test. All blends of probiotic strains with Pectin as prebiotic material shown significant increase of biomass as compared to single probiotic administration. All results suggest the co-administration of Pectin with the L. acidophilus L. plantarum, B. bifidum, and B. lactis probiotic strains enhances in vitro and in vivo viability of these bacteria. More thorough quantitative analysis of the extract for in vivo parameters can help utilising it in the nutraceutical formulations as a prebiotic compound or as a synbiotic composition along with selected probiotic strains.



Keywords: Synbiotics, Prebiotic Potential, Pectin, Probiotic Bacteria

INTRODUCTION

Prebiotics are defined as non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon [1]. Prebiotics are the substrates are fermented or utilised by the gut by bacteria i.e., probiotic bacteria, producing the postbiotics like short-chain fatty acids (SCFA) among which acetic acid, propionic acid and butyric acid are the most important. SCFA concentrations are implicated in colonic diseases, like cancer, inflammatory bowel disease, immunomodulation, etc. [2, 3]. Changes in the quantity of different genus could affect the physiology and pathology of the hosts [4]. Prebiotics are usually found in diverse food sources, such as chicory, chia seeds, dandelion greens, flaxseeds, onion, garlic, almonds, artichoke, oats, barley, and many other plants, although they can also be synthesized via enzymatic digestion of complex polysaccharides.

Pectin is a heteropolysaccharide contained in the primary cell walls of terrestrial plants. It is a multifunctional constituent of cell wall is a high value functional food ingredient extensively used as gelling agent and as stabilizer [5]. Commercially, it is produced in the form of white to light brown powder, mainly extracted from citrus fruits, and is used in food as a gelling agent particularly in jams and jellies. Similarly, it is used in fillings, sweets, as a stabilizer in fruit juices and milk drinks and as a source of dietary fiber**.** In human digestion, it goes through the small intestine more or less intact but is acted upon by microbial growth of large intestine, thus acts as a soluble dietary fibre [5]. In the large intestine and colon, microorganisms degrade pectin and liberate short-chain fatty acids i.e. prebiotic effect [6]. It is reported to have antilipidemic, antiulcerogenic, antilithiatic, antimicrobial, analgesic, antihypertensive, anti-diarrhoeal, antiallergic, antioxidant, diuretic, hypolipidemic, hypoglycaemic, hair growth promoting, haemostatic, muscle relaxant, mutagenic, wound healing and vasodilatory activities [6]. It is finding increasing applications in the pharmaceutical and biotechnology industry. It has been used successfully for many years in the food and beverage industry as a thickening agent, a gelling agent and a colloidal stabiliser. Pectin also has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of a variety of drugs, proteins and cells [6]. Nagpur mandarin (*Citrus reticulata* Blanco) is one of the important fruits grown in central India, occupying 40 % of the total area under citrus cultivation [7]. *Citrus reticulata* is underutilised than its production rate in nutraceutical values. Pectin can be isolated from the waste peels of this orange variety from juice companies for its multiple uses including prebiotic application. It is also an environment friendly approach to avoid making piles of food wastes. Thus, this plant is selected in present work as a source of prebiotic.

Gut microflora especially, bacteria play an significant role in human health, such as supplying essential nutrients, synthesizing vitamin K, aiding in the digestion of cellulose, and promoting angiogenesis and enteric nerve function. Though, they can also be potentially harmful due to the change of their composition when the gut ecosystem undergoes abnormal changes in the light of the use of antibiotics, illness, stress, aging, bad dietary habits, and lifestyle. Dysbiosis of the gut bacteria can result in many chronic diseases, such as inflammatory bowel disease, obesity, cancer, and autism [8]. *Bifidobacterium* and *Lactobacillus*, are most common species of probiotic bacteria, assigned to have multiple health benefits [9, 10].

Present research work is composed of isolating pectin from *Citrus reticulata* fruit peel and developing a synbiotic formulation along with lyophilised *Lactobacilli* and *Bifidobacteria* to study the improved viability while passing through gastric environment.

MATERIAL AND METHODS

Pectin isolation

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 dried pectin was calculated.

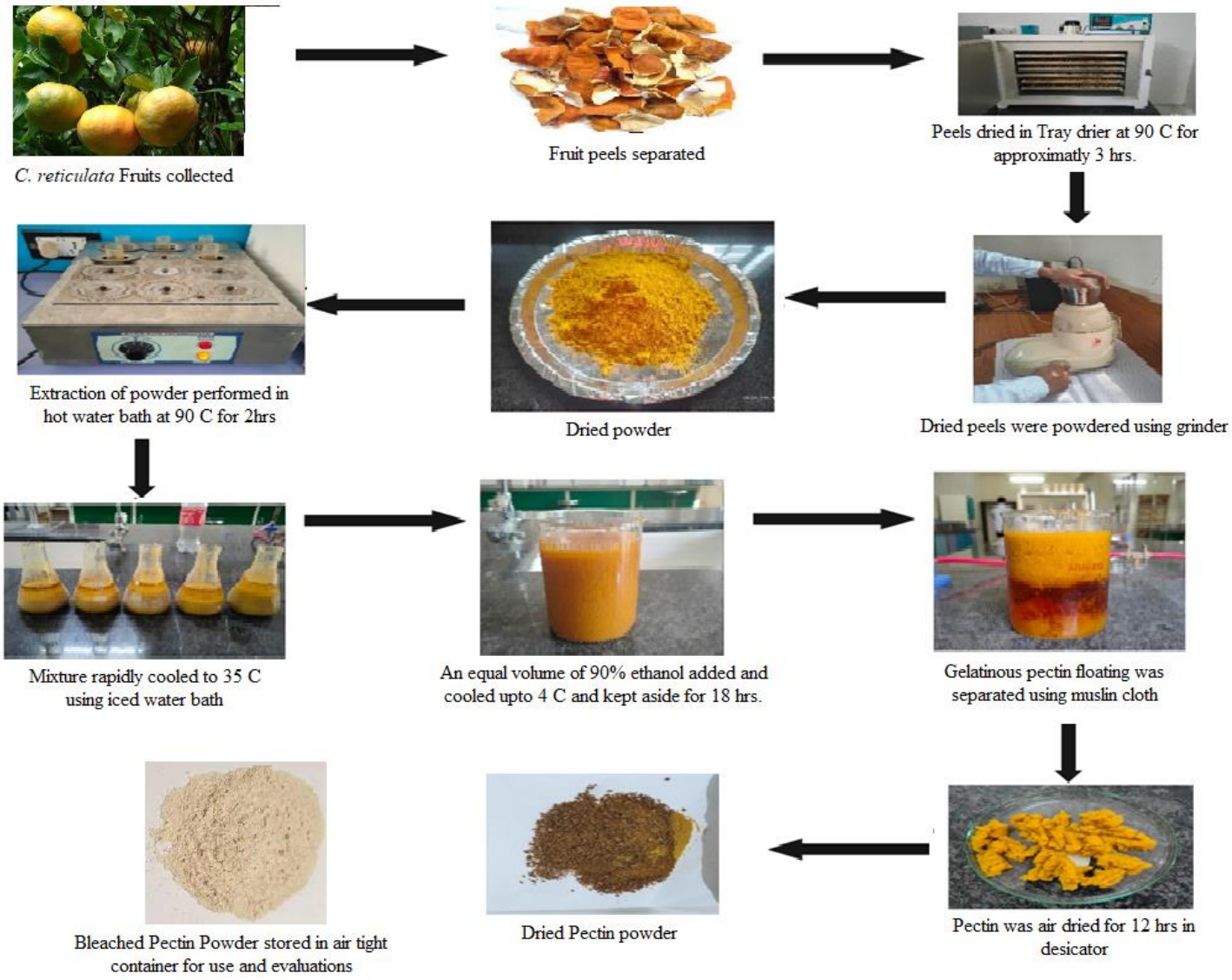
Evaluation of extracted pectin

Extraction yield of isolated pectin was determined using equation 1.

$$\text{Yield} = \frac{\text{Weight of dried pectin (g)}}{\text{Weight of dry orange peel Taken for extraction (g)}} \times 100 \quad \text{- equation 1}$$

Pectin was evaluated for moisture content, solubility, total ash value, and gelling property according to standard methods. The comprehensive phytochemical analysis was performed to evaluate the essential constituents in Nagpuri orange pectin, including test for carbohydrates, proteins, phenolic compounds, and flavonoids [12–14]. Total phenolic content was measure using Gallic Acid Equivalent (GAE) method [15–17] and total flavonoid content was measured following spectrophotometric method [17, 18].

Figure 1: Pictorial presentation of the Pectin extraction process



Probiotic Strains

Lyophilised Probiotic bacterial strains *of L. plantarum* (LP), *L. acidophilus* (LA), *B. bifidum* (BB), and *B. lactis* (BL), were kindly provided by Unique Biotech Pvt. Ltd., Hyderabad were used as probiotic model bacteria in the experiment described. This strain has been reported as a probiotic in previous studies [19, 20]. The number of probiotic cells in the granules ranging from 10^{10} to 10^{11} cfu/g. Lyophilised bacterial cells were revived by growing in Man de Rogosa and Sharpe (MRS) broth (Himedia, India) at 37°C under anaerobic conditions for 24 hours. These were sub-cultured weekly for assuring regular supply for analytical studies during study in MRS broth. Stock culture was stored at - 80°C in 20% (w/v) glycerol for further use. A percentage of 0.05% of L-cysteine was added to commercial MRS broth in order to improve anaerobic conditions and stimulate the growth of the probiotic [21, 22]. The isolated colonies formed on the 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 bacteriology [23].

Synbiotic Characterisation

Test Tube method

Frozen stock culture of the microorganism was sub-cultured overnight in vials with 10 ml of modified MRS broth with the corresponding carbon sources and incubated for 12 hrs at 37°C under anaerobic conditions. The assays included a negative control without carbohydrate (basal medium), a positive control with glucose as optimal carbon source, inulin as a reference prebiotic, and the test

sample of Pectin. To prepare the medium, the glucose, and Pectin were first dissolved in basal broth then autoclaved for 15 min. at 110°C. Pectin and glucose were sterilized before being added to the culture media [24, 25]. MRS media containing a 1.5% concentration of Pectin was inoculated with a prewashed 1% inoculum of LP, LA, BB & BP probiotic bacterial strains in separate tubes ($1x10^9$ cfu/ml). These tubes were incubated in micro-aerophilic conditions overnight at 37°C under appropriate atmospheric conditions for a period of 48 hrs. Optical density, pH, titratable acidity, SCFAs content, and biochemical tests were performed to verify the viability of bacterial cells.

Optical Density

Growth of each strain was monitored separately by measuring the optical density (OD) using UV spectrophotometer (UV-1201 Shimadzu) at wavelength of 600 nm for the cultures at intervals of 0, 4, 8, 12, 24, 28, 36 and 48 hrs. At each test point, 5 ml of fermentation broth was removed and the optical density (OD) was measured at wavelength of 600 nm. Based on the results of these studies the postbiotics to be tested in the 48 hrs. The maximum specific speed for the growth was calculated during the exponential growth phase through the following equation 2 [26].

$$\mu_{max} = \frac{\ln(OD_{max.}) - \ln(OD_{min.})}{T} \quad \text{--------- Equation 2}$$

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 doubling time was determined through equation 3 -

$$td = \ln 2/\mu_{max} \quad \text{---------- equation 3}$$

Where,

μ_{max}- maximum specific growth rate,

td- doubling time

pH change

The pH was measured by an electronic digital type pH meter (EquipTronic, India). pH 4.0 and pH 7.0 buffer solutions were used to standardize the pH meter.

Titratable Acidity

Media containing cultures and metabolic acids were titrated after 48 hrs with 0.01 M NaOH using phenolphthalein as an indicator [27–29]. The percent titratable acidity of each combination was calculated by using equation 4:

$$\% \text{ Titratable acidity} = \frac{9 \times \text{Titer value} \times \text{normality of NaOH} \times \text{Dilution}}{\text{Weight of the sample taken}} \times 100 \quad \text{-----equation 4}$$

SCFA Detection

Colorimetric method is used for detection of acetate and butyrate produced in the culture test tubes was determined by UV spectrophotometry [30–32]. The grown culture was diluted with PBS and centrifuged to separate the bacterial cells. Supernatant fluid was separated for determining the SCFAs present. Calibration curve was obtained using standard butyrate, acetate and propionate using UV spectrophotometer (UV-1201 Shimadzu) in the range of wavelength 200 to 260 nm for acetate [33]

Biochemical Tests

Biochemical tests were performed on the basis of carbohydrate fermentation test, motility test, catalase and oxidase test, for confirming type of probiotics strains grown [34,35].

Validation of Test Tube Method by Plate Count Methods

Inoculum containing probiotic bacterial strains and Pectin were separately diluted with Phosphate Buffer Solution (PBS) (Hi-media Pvt. Ltd.) and plated on MRS 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 [36]. Colonies were enumerated and the results were expressed as colony forming units per gram. Colonies were identified by colony morphological characteristics and the identification was confirmed by biochemical tests.

Characterization of Colonies

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, and presence of flagella were observed to confirm the type of Bacteria [36].

In vivo prebiotic potential Pectin

Amongst different probiotic strains Lactobacillus strains i.e., LA and LP shown comparatively good in vitro blend to consider as synbiotic combination for the in vivo evaluations. Therefore, these two samples were assessed *in-vivo* in a 6 weeks old female Sprague Dawley Rat. Rats had free access to diet and water and were housed in ventilated room with a 12hr: 12hr light/ dark photoperiod at 23 ± 2ºC. All procedures had approval of the Institute Animal Ethical Committee. After a week of acclimatization to experimental conditions, the rats were randomly divided into three groups (n = 6). Control group I received distilled water; Group-II, and III received LP+ Pectin and LA+ Pectin (2g/day/kg of rat) respectively suspended in distilled water for 4 weeks. Stool samples were collected from each animal every week after starting the treatment, and immediately stored at 4°C. Freshly voided faecal material was collected to study faecal moisture, pH and bacterial concentrations. The water content of the luminal stools was calculated by weight difference between fresh and dried (kept during 24 hrs. at 65ºC) samples. For pH measurement, faecal content was suspended in water, homogenized by vortexing and

pH values was measured using a pH-meter (Equip Tronic, India). At the end of 4th week animals were sacrificed, transferred to laminar flow cabinet and caecum content was removed anaerobically. pH and bacterial count of caecum content was determined. Ten times serial dilutions were made in the medium and aliquots of 0.1 ml of the appropriate dilutions was spread onto the MRS agar media to determine bacterial count; MRS agar for lactobacilli (Himedia, India) supplemented with 0.5 g/l L-cysteine hydrochloride and culture plates were incubated micro-aerophicaly for Lactobacilli at 37°C for 24–48 hrs [37].

Microbiological Analysis of Caecum Content

For the isolation and counting of probiotic bacteria each one of the caecum samples were aseptically diluted by 0.1% (w/v) sterile peptone water. Aliquots of these suspensions were transferred to test tubes containing 9 ml with the same peptone water, so as to obtain serial decimal dilutions (10^{-1} to 10^{-6}). Later, an aliquot of each diluted sample was transferred to plate dishes containing modified MRS agar as described previously, the counting was performed by a colony counter apparatus. (Labline, India)

Statistical Analysis

For each experiment, the data was analysed using the Excel statistical package. Statistical analysis was analyzed by using One-Way analysis of variance (ANOVA) which shown significantly difference with $p < 0.05$.

RESULTS

Isolated pectin was light yellowish in colour, soft amorphous powder with characteristic citrus smell. Pectin extraction yield obtained was expressed as dry weight and was calculated as 22.5 % w/w of orange peel powder. Moisture content was 4.2 %, total ash was found as 5.4 % w/w.3.2% w/v pectin in water is mixed to receive a homogeneous solution. Boiling and cooling of 5 % w/v of pectin in water shown jelly formation.

The comprehensive phytochemical analysis highlights the abundance of essential constituents in Nagpuri orange pectin, including carbohydrates, proteins, phenolic compounds, and flavonoids. Specifically, the total phenolic content measured at 1.82 ± 0.02 mg Gallic Acid Equivalent (GAE)/g of extract and the flavonoid content at 1.39 ± 0.015 mg GAE/g of extract signify a distinctive composition that may contribute to observed health benefits.

All the stains of prebiotic bacterials shown satisfactory growth in MRS media with the administration of Pectin as a sole carbon source as mentioned in Graph in Figure 2. In the presence of Pectin, growth curves demonstrated significant increases in optical density (OD), with the maximum OD observed for LA at 2.12 ± 0.04 cfu/g in modified MRS medium and for LP at 1.56 ± 0.04 cfu/g. Values indicates the proficiency of LA in both the media. Optical density indicated the enhanced bacterial growth in all Medias with Pectin as shown in Table 2.

Figure 2: Growth curve of probiotic strain in MRS media using Test Tube method

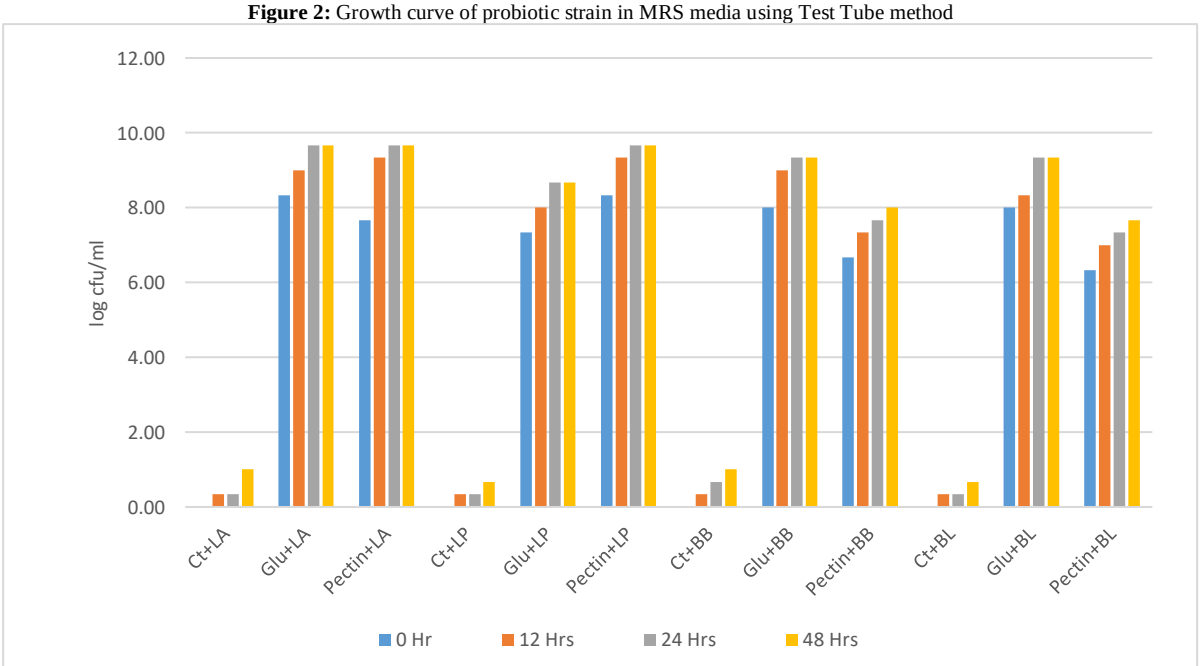


Table 1: Titratable acidity of MRS mediums containing different probiotic strains supplemented with Pectin after 48 hrs.

Sample →	Control	LA	LP	BB	BL
MRS + Pectin	0.09 ± 0.04	2.12 ± 0.04	1.38 ± 0.03	0.48 ± 0.05	0.68 ± 0.04
RCM + Pectin	0.09 ± 0.04	1.56 ± 0.04	1.22 ± 0.04	1.38 ± 0.03	1.54 ± 0.05

Note: The values represent the average cfu/g ± S.D. (n = 3), $p < 0.05$

Table 2: in vitro cfu/gm MRS mediums containing different probiotic strains supplemented with Pectin using test tube method and validated using plate count method

Sample →	Control	LA	LP	BB	BL
Test Tube method	0.09 ± 0.04	2.12 ± 0.04	1.38 ± 0.03	0.48 ± 0.05	0.68 ± 0.04
Plate count method	0.09 ± 0.04	1.86 ± 0.04	1.22 ± 0.04	0.5 ± 0.03	0.64 ± 0.05

Note: The values represent the average cfu/g ± S.D. (n = 3), $p < 0.05$

Table 3: Growth kinetics of Probiotic strains in presence of Pectin in test tube method

Sample	Control	Pectin +LP	Pectin +LA	Pectin + BB	Pectin + BL
µmax (h-1)	0.173	0.028	0.030	0.048	0.048
Doubling Time (td) h	4.006	24.75526	14.44057	23.10491	23.10491

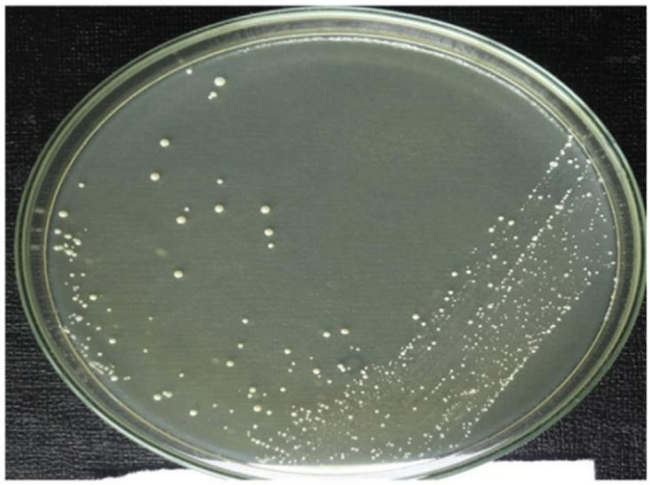
Table 4: Colony characterisation for isolated colonies of in vivo faecal samples through plate count method.

Groups		Control	LA + Pectin	LP + Pectin	BB + Pectin	BL + Pectin
pH		6.8 ± 0.6	6.05 ± 1.32	6.19 ± 1.21	6.7 ± 0.4	6.7 ± 0.2
% Water content		72.5 ± 1.12	54.2 ± 1.12	66.4 ± 1.58	70.5 ± 1.1	71.1 ± 1.2
Log cfu/g of wet caecum content		8.04 ± 0.03	8.92 ± 0.04	8.04 ± 0.03	8.23 ± 0.01	8.4 ± 0.02
Colony characterisation for the presence of bacterial colonies	Phenotypic characteristics	+ve	+ve	+ve	+ve	+ve
	Microscopic observation	+ve	+ve	+ve	+ve	+ve
	Gram's Staining	+ve	+ve	+ve	+ve	+ve
	Spore formation	-ve	-ve	-ve	-ve	-ve
	Carbohydrate fermentation	+ve	+ve	+ve	+ve	+ve
	Catalase reduction	-ve	-ve	-ve	-ve	-ve
	Lactic acid fermentation	+ve	+ve	+ve	+ve	+ve
	Arginine hydrolysis Test	+ve	+ve	+ve	+ve	+ve

Note: The values represent the average ± S.D. (n = 3).

Figure 3: In vivo Colony isolation from LP + Pectin

Figure 4: In vivo Colony isolation from LA + Pectin



Addition of Pectin significantly reduces pH of modified MRS medium. Reduction in pH from near neutral range i.e. approximately 6.8 - 6.9 (t = 0 hr) to 6.1 - 5.5 (t = 48 hrs) with all the combinations indicated strong relationship to production of acids like lactic acid which is main metabolic product produced by the *Lactobacilli*. pH of the media shown decline due to the release of metabolic acids in the media as metabolic byproducts by the probiotic strains.

Declined pH indicate the presence of lactic acid, butyric acid, propionic acid, and some other postbiotics as they fall under the observed pH range. Thus, the percent titratable acidity was determined, which was observed increased in all the Medias containing different strains due to presence of acid in the media after 48 hrs as shown in table 1.

Test tube method results were attempted to get validated with the plate count method. It is compatible with the ability of Pectin as prebiotic source for selected bacterial strains. In vitro cfu/gm of modified MRS medium supplemented with Pectin added different probiotic strains explained in Table 2.

Lag phase of *L. acidophilus* growth curve was decreased, and log phase was increased significantly after addition of Pectin, as sole carbon source to modified MRS medium (Figure 1). Then, bacterial growth curve reached to stationary phase after 32 h and turned to be relatively constant for 48 h Growth kinetics the maximum specific growth rates (μ_{max}) and the doubling time (td) of the media

containing different carbon source after 48 h of anaerobic fermentation are shown in Table 3. L. acidophilus showed the fastest growth (µmax, 0.173 h-1) on MRS added with Pectin compared to without Pectin (µmax, 0.028 h-1).

The results of plate count method are complimentary to the test tube method. Thus, both test tube method and plate count method results validate each other for the growth enhancement of selected probiotic strains of bacteria with the supplementation of pectin.

Microscopically they were Gram-positive, rod shaped, non-motile, non-sporulating, catalase negative, and shown positive results for carbohydrate fermentation, lactic acid and arginine hydrolysis test.

In vivo studies further validated these findings, illustrating the positive impact of Pectin on rat faecal samples. In vivo isolated colonies from rat fecal content through plate count method are shown in figure 3 & 4. The growth kinetics of L. acidophilus further provided quantitative insights, demonstrating increased growth rates and reduced doubling times in the presence of Pectin.

This research collectively advances our understanding of Pectin as a promising prebiotic candidate derived from *C. reticulata*. It lays a foundation for potential applications in functional foods or supplements aimed at enhancing gut health. Future investigations could delve deeper into the mechanistic aspects of Nagpuri orange pectin's prebiotic effects, exploring its application in diverse food and health contexts for a more comprehensive perspective.

List of Abbreviations

Abs- Absorption

ABTS- 2.2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

ANOVA- analysis of variance

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- deMan Rogosa Sharpe media

MTT- 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

Nm- Nano meter

OD- optical density

Pvt. Ltd-Private limited

QE- quercetin equivalents

RCM- Reinforced clostridial medium media

SCFA- short-chain fatty acids

td- doubling time

UV-Ultra violet

W/w- Weight by weight

CONCLUSION

Pectin is a polysaccharide possessing nutritive value for the probiotics bacterial strains is proved in the present work. It can be isolated from different varieties of oranges but *Citrus reticulata* can serve as a source for it as it's produced in middle India on large scale with huge amount of waste byproducts. Synbiotic combinations studies in present work like Pectin with the *L. acidophilus L. plantarum, B. bifidum, and B. lactis*, are showing better viability while passing through gastric environment. All results suggest the coadministration of probiotic strains enhances in vitro as well as in vivo viability of these bacteria. The study also reveals the effective validation of test tube method with the plate count method for measuring the prebiotic potency of any material. More thorough quantitative analysis of the extract for in vivo parameters can help utilising it in the nutraceutical formulations as a prebiotic compound or as a synbiotic composition along with selected probiotic strains.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the IEAC, P. Wadhwani College of Pharmacy, Yawatmal approved under 650/PO/Re/S/2002/CPSCEA.

Conflicting of interests

The authors declare that they have no conflicting interests.

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