



Synergistic Effects of Okara-Based Prebiotic and *Lactobacillus plantarum* Probiotic: Development of A Nutraceutical Formulation for Gut Health

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Abstract

Probiotics and prebiotics collectively known as synbiotics enhance gut health and microbial activity. The study attempted to develop a freeze-dried synbiotic nutraceutical formulation with Okara as a prebiotic and *L. plantarum* as a probiotic to improve gut health while preserving beneficial microbes. *L. plantarum* was morphologically characterized using biochemical assays, and enzymatic profiling was performed with VITEK 2C. Okara's protein content and ash levels were examined to evaluate its pharmacognostic characteristics and prebiotic efficacy was assessed at 1%, 2%, 3%, and 4% concentrations, emphasizing specific growth rate, pH, % titratable acidity, and dry biomass. Nine batches of a synbiotic powder formulation containing cryoprotectants such as mannitol, sorbitol, and maltodextrin, with probiotics and prebiotics, were freeze-dried. Three of the nine batches have been selected, with B2 selected for further examination. *L. plantarum* viability was evaluated before and after freeze-drying and storage at 4-8 °C. The prebiotic efficacy test demonstrated that 2% okara resulted in significant growth, indicating significant *L. plantarum* proliferation. After 48 hours, the pH declined to 4.24, and the percentage titratable acidity gradually increased, indicating significant lactic acid production. The dry biomass content was maximum at 2% okara. Following freeze-drying, viable counts decreased to 3.78 ± 0.045 log CFU/mL and 3.54 ± 0.015 log CFU/mL after 7 days of storage. Batch B2 demonstrated



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excellent flow properties, smaller particle size with minimum drying loss. At 48 hours, the zone of inhibition against *E. coli* was demonstrated to be highly susceptible. *L. plantarum* counts increased to 9.68 ± 0.19 log CFU/mL after 48 hours in the in vitro co-culture investigations with *E. coli*. However, the number of *E. coli* bacteria decreased significantly. The combination demonstrated remarkable effectiveness in enhancing *L. plantarum* proliferation while suppressing *E. coli*-, indicating plausible uses in nutritional supplements for promoting gut health.

Abbreviations

% TA: % Titratable Acidity; **ANOVA:** Analysis of Variance; **BSA:** Bovine Serum Albumin; **CFU -** Colony Forming Unit; **Doubling Time:** Dt; ***E. coli:*** *Escherichia coli*; **FOS:** Fructooligosaccharide; **H₂O₂:** Hydrogen Peroxide; ***L. plantarum:*** *Lactobacillus plantarum*; **MEM:** Minimal Essential Medium; **MRS:** de Man, Rogosa, and Sharpe; **NaOH:** Sodium Hydroxide; **ns:** Non-significant; **OD:** Optical Density; **SCFAs:** Short-Chain Fatty Acids; **SEM:** Standard Error of the Mean; **TSI:** Triple Sugar Iron; **USP:** United States Pharmacopeia; **Zone of Inhibition:** ZOI

Introduction

The intestine is a habitat for diverse microorganisms that regulate intestinal homeostasis and immunological functions.^{1,2} The gut microbiota and intestinal immunity are inextricably connected, with a healthy microbiome promoting immune system development and function^{3,4} by the production of microbial metabolites such as short-chain fatty acids (SCFAs).^{5–12} Prebiotics are defined as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of bacteria in the colon and thus improves host health".^{13–20}

The research on the synergistic effects of okara-based prebiotics and *L. plantarum* probiotics highlights a promising yet underexplored area in gut health. Okara, or soybean residue, is a by-product of the tofu and soymilk manufacturing process. Despite its high quantity of nutritional fiber, protein, and beneficial components, it is often overlooked and thrown as waste. It is high in dietary fiber, specifically soluble and insoluble fibers and oligosaccharides, as well as proteins and other bioactive components such as isoflavones and antioxidants. The oligosaccharides in the okara serve as substrates for healthy gut flora, promoting

their growth and activity. It holds immense potential as a prebiotic.^{21–24} These assist in protecting the gut lining from oxidative damage and inflammation. Okara additionally serves by lowering inflammation in the stomach and controlling immunological response, and it confers health advantages to hosts.^{25–32} Researchers reported the prebiotic effect of okara by carrying out *in-vitro* studies using *L. acidophilus* and *Bifidobacterium bifidum*,^{33,34} and it was noted that okara provided a surface for the adhesion of cells, which ultimately enabled the uptake of the substrate and cell growth. Furthermore, insufficient research has been discovered regarding the precise mechanisms by which okara-derived prebiotics and *L. plantarum* interact, particularly their ability to improve probiotic viability, modify the composition of the gut microbiota, and improve overall gastrointestinal health. The current study addresses this research gap by integrating the probiotic efficacy of *L. plantarum* with the prebiotic potential of okara.

L. plantarum is a potential probiotic lactic acid bacterium commonly found in various fermented foods and the human gastrointestinal tract.^{35–37} According to research, *L. plantarum* strains have exceptional resistance to bile salts and hydrochloric acid (pH 2.0), with survival rates varying from 0.003 to 10%, in contrast to other strains such as *L. sakei* and *L. paracasei*.³⁸ *In vivo* investigations have also demonstrated that *L. plantarum* has a high survival rate subsequent to human consumption. For instance, after a single injection of 108 cells, *L. plantarum* NCIMB 8826 had a 77% survival rate in the human ileum, much surpassing other strains like *Lactococcus lactis* and *Lactococcus fermentum*.³⁹ Furthermore, *L. plantarum* can survive in the human gut for up to 11 days adhering to consumption, with colonization rates varying by individual.⁴⁰ *L. plantarum*'s ability to attach to human cells, particularly via mannose-inhibited binding, is crucial

for its survival and probiotic function. It may also protect the host against pathogenic infections.^{41,42} It possesses various probiotic qualities important for gut health, including resistance to acidic conditions in the stomach and bile salts in the intestine, improving gut survival and colonization. It also produces anti-inflammatory chemicals and modulates immunological responses, which reduce intestinal inflammation. The research aims to develop a synergistic synbiotic nutraceutical formulation containing *L. plantarum* as the probiotic and okara as a prebiotic high in dietary fiber. This composition attempts to increase the viability and stability of beneficial microbes, eventually promoting gut health by inhibiting the development of pathogenic bacteria. To accomplish this, the study focuses on improving the encapsulation of *L. plantarum* within okara, assuring the probiotic's viability and efficacy. The formulation was further developed by evaluating the physicochemical parameters of freeze-dried powder formulation.

Furthermore, the stability of *L. plantarum* was investigated demonstrating the formulation's viability and efficacy in maintaining a healthy gut microbiome.

Materials and Methods

Materials

L. plantarum was isolated from cabbage, a vegetable source. One gram of cabbage was suspended in saline, vigorously mixed, and centrifuged at 300 g to settle the particle matter. The supernatant was serially diluted tenfold with saline, and 100 μ L of the 10^{-7} dilution was plated on MRS agar. The plates were then incubated at 30°C in microaerophilic conditions for 24-48 hours.⁴³ Mprex Healthcare Pvt. Ltd. Waked, Pune, provided gift samples of okara (Soyabean waste). *Lactobacillus* de Man Rogosa and Sharpe (MRS) agar, MRS broth, McConkey agar, McConkey broth, fructooligosaccharide (FOS), sorbitol, mannitol, and bovine serum albumin (BSA) was acquired from HiMedia, Mumbai, India. Maltodextrin was sourced from Lobachemie, Mumbai, India. All reagents utilized in this investigation were high purity and fulfilled the analytical standards of the experimental techniques.

Phenotypic Characterization of *L. plantarum*

Characterization of *L. plantarum*

The *L. plantarum* was cultured on an MRS agar plate using the streak plate technique and incubated

at 37°C for 48 hr. The colony morphology, shape, colour, elevation, margin, and other notable features were examined. Gram staining was performed on a clean, grease-free slide to study the staining morphology. A smear of suspension was created, which was then heat-fixed onto the slide. The initial staining was performed with a water-soluble crystal violet dye, grams of iodine as a mordant, 95% ethyl alcohol as a decolourizing agent, and safranin as a counterstain.^{44,45} After drying, the smear was examined using an oil immersion objective.

Catalase Test and Oxidase Test

One small amount of well-isolated *L. plantarum* colony was mounted on a microscope slide. One drop of 2% H_2O_2 was added to the bacteria immediately and covered with a cover slip. The formation of effervescence was observed after the addition of H_2O_2 . Furthermore, the oxidase test was performed to determine whether the isolates could utilize cytochrome C oxidase, an enzyme in the electron transport chain.⁴⁶ An isolated bacterial colony was transferred to a sterile Kovacs oxidase reagent (tetramethyl-p-phenylene-diamine dihydrochloride) oxidase test disc.⁴⁷ The findings were reported based on colour changes; the formation of a deep purple/blue colour indicates oxidase production, while no change in colour indicates negative results.

TSI (Triple Sugar Iron) Test

The TSI medium contained three sugars (lactose, sucrose, and glucose) and iron, which are utilized to confirm the bacteria's capacity to utilize these sugars. TSI agar slants were prepared and inoculated with *L. plantarum* by stabbing through the butt of the medium, then streaking the slants. The inoculated slant was kept at 37 °C for 18-24 hours. Following incubation, sugar fermentation, gas production, and hydrogen sulphide production were all examined with color change and gas production.^{46,48}

Identification using the Vitek 2C System

The colonies of lactobacilli from MRS agar plates were identified using the Vitek 2C system (VITEK 2 Systems Version 9.2) following a preliminary screening. The ANC database includes a collection of 63 different types of anaerobic bacteria, including *Lactobacillus*.^{49,50} The individual lactobacilli colonies were selected from MRS agar plates and suspended in normal saline, exhibiting turbidity ranging from 2.7 to 3.3 McFarland units. The inoculums

were introduced into an ANC (Anaerobic and Corynebacteria identification) card in the Vitek 2C system and incubated for approximately 6 hours. The identification process is initiated by examining microbial growth patterns and biochemical responses to determine the species and provide interpretations for final identifications.⁵⁰

Characterization of Okara

The pharmacognostic profiles of okara were evaluated, including total ash value, water-insoluble ash value, and acid-soluble ash value. The total ash value was determined by utilizing pre-weighed 2 g of okara incinerated in the furnace until constant weight was obtained. The water-insoluble ash value was examined using distilled water, and the acid-soluble ash value was determined using 10% HCl.⁵¹ The phytochemical screening procedures of okara comprise the molish test for carbohydrate content, the biuret test for protein content, and the bromine water test for tannin content.

Bradford Assay

The Bradford assay determined the protein content of the okara in comparison to BSA, which was used as the reference standard to plot a calibration curve using known concentrations.⁵² The okara and BSA solutions were prepared, resulting in stock solutions with a concentration of 2000 µg/mL in distilled water. To achieve concentrations of 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL, 1000 µg/mL, and 1200 µg/mL, samples were collected from test solutions and diluted with distilled water to a final volume of 10 mL. Bradford dye solution (Coomassie brilliant blue G-250) (5 mL) was added to each diluted sample, and the solutions were incubated for 5 to 10 min. The absorbance was measured at 595 nm after incubation.⁵³

Prebiotic Efficacy of Okara

The growth kinetics of *L. plantarum* were evaluated in reconstituted MRS broth supplemented with okara and FOS and containing tryptone, pectin, meat extract, potassium phosphate dibasic, sodium acetate, tri-ammonium citrate, and magnesium sulphate. *L. plantarum* cultured in MRS broth was utilized as the blank sample. To cultivate *L. plantarum*, 2% (v/v) inoculum was added to 50 mL of sterile, reconstituted MRS broth under aseptic conditions. The flasks were subsequently placed

in an orbital shaking incubator (Remi electro tech, CIS 18, India) and incubated for 48 hours at 37± 2 °C. Samples were collected after 0, 2, 4, 6, 24, and 48 hours, and the optical density (OD) at 600 nm was recorded. The growth rate (µ) was calculated during the exponential phase of growth using the following formula,

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t_2 - t_1}$$

Where,

µ = growth rate (h⁻¹)

N_t = Bacterial population at time t₂

N₀ = Bacterial population at time t₁

t₂ - t₁ = time interval between the two measurements

The doubling time (g) was calculated from the growth rate using the following formula,

$$g = \ln(2)/\mu$$

A cooling centrifuge (Remi electro tech, C-24 plus ZFCU-07420, India) was used to centrifuge the culture at 1000 rpm to produce a cell-free supernatant for further analysis. The bacterial pellet that remained over after the centrifugation was utilized to evaluate the dry biomass.

Determination of Dry Biomass

To monitor the cellular development process, a cooling centrifuge (Remi electro tech, C-24 plus ZFCU-07420, India) was used to separate the developed bacterial cells pellet by centrifuging the sample at 1000 rpm for 30 minutes. The separated cell pellet was dried in a hot air oven at 40 °C (Remi Electro Tech, AI-7982, Mumbai, India) until it reached a consistent weight as dry biomass.

Determination of pH and Titratable Acidity

The pH of the cell-free supernatant was determined using a calibrated electronic digital pH meter (EQ-614A Equip-Tronic, Mumbai, India) at each time interval. This method indicates organic acid generation and is essential for determining the *Lactobacillus* probiotic and fermentative characteristics.

The % Titratable acidity of *L. plantarum* was measured by titrating the bacterial cell-free supernatant with 0.1 M NaOH and using phenolphthalein as an indicator. The titratable acidity was calculated as follows,

$$\% \text{ Titratable acidity} = (N \times V \times M) / (S \times 10)$$

Where, N is the Normality of NaOH, V is the Volume of NaOH used for titration (mL), and M is the Molecular weight of the predominant acid in the sample divided by the number of hydrogen ions in the acid molecules that are titrated, S is the Sample size in mL or g.

Determination of Anti-Microbial Activity

The antibacterial activity of cell-free supernatant from *L. plantarum* culture was assessed against *E. coli* using the agar well diffusion technique using MacConkey agar. The overnight cultures (0.1 mL) of *E. coli* were evenly distributed on agar. The wells were cut to a diameter of 6mm, and each well received 0.1 mL of supernatant. After allowing the sample to diffuse for 4 hours in the refrigerator, plates were incubated at 37 °C for 24 hours under aerobic conditions. The zone of inhibition was calculated.

Formulation of Freeze-Dried Powder

The formulation was performed on a laboratory-size lyophilizer (Martin Christ, Alpha 2-4LSC, An der Unteren Söse, Germany). The formulation was optimized for the carrier type (mannitol, sorbitol, and maltodextrin) and carrier-to-prebiotic ratio (0.5:1, 1:1, 2:1), as shown in the table. Okara was used as a prebiotic, with mannitol, sorbitol, and maltodextrin serving as cryoprotectants for lyophilization. *L. plantarum* was utilized as a probiotic at 1.2×10^9 CFU/mL. The samples were lyophilized for 30 minutes for freezing, warming for 10 minutes, primary drying for 4 hours (vacuum 0.0050 m pressure), and final drying for 3 hours (vacuum 0.1000 m bar). The process was continued for two days to eliminate any residual moisture.

Formulation of Synbiotic Freeze-Dried Powder

Nine batches were evaluated for powder yield and micromeritic parameters. These investigations were performed to select the batch with the highest yield and optimal micromeritic properties. Each batch underwent evaluation under the same conditions, and the results were compared to determine the

optimal powder yield and appropriate micromeritic characteristics.

Powder Yield and Micromeritic Properties

The yield of freeze-dried powder was determined by dividing the weight of freeze-dried powder by the theoretical weight of solids before freeze-drying.

$$\text{Yield (\%)} = (\text{Weight of freeze dried powder (g)} / (\text{Weight of solids before freeze drying (g)} \times 100$$

The micromeritic properties of the freeze-dried powder were evaluated in compliance with the USP 2018 standard methodology, including the Bulk density, Taped density, Carr's index, Hausner's ratio, and Angle of repose.

Particle Size

Optical Microscopy

Particle size was measured in freeze-dried formulations using optical microscopy and a calibrated eyepiece micrometer. The freeze-dried powder on the slide and the size of 100 particles were measured under the microscope using the eyepiece scale, which was divided into 100 minor divisions. Each division is equivalent to 10 meters. The arithmetic and geometric mean diameters of the particles were calculated.

Laser Diffraction

Laser diffraction (Malvern Meta sizer) was used to measure the particle size of the optimized freeze-dried powder batch. A cumulative undersize was created, allowing the identification of granule sizes corresponding to the 10th, 50th, 90th, and 100th percentiles (D10, D50, D90, and D100). The calculation was expressed in terms of arithmetic mean diameter.

Evaluation of Reconstituted Powder

Viability Enumeration

The formulation's viability was tested before and after freeze-drying by spreading it on MRS agar plates. The results are expressed in CFU/mL using mean and standard deviation. The viability of freeze-dried powder was determined by performing ten-fold serial dilutions of reconstituted powders on MRS agar plates. The reconstituted powder was serially diluted with MRS broth before plating. The MRS plates were incubated at 37 °C for 48 hours, and the bacterial colonies were counted.

In-vitro Efficacy using the Co-Culture Technique

Minimal Essential Medium (MEM) was prepared, which included yeast extract, tween 80, tryptone, meat extract, potassium phosphate dibasic, sodium acetate, triammonium citrate, and magnesium sulphate. The media was then inoculated with 0.5 MacFarland standards of *E. coli*. Several flasks were prepared: flask 1 contained the control *E. coli*, flask 2 contained the formulation and *E. coli*, flask 3 contained *L. plantarum*, and flask 4 contained *L. plantarum* with formulation and *E. coli*. The flasks were incubated for 48 hours. At 0, 6, 24, and 48 hours of incubation, the sample was withdrawn from each flask and added to the sterilized broth to make serial dilutions (10^{-8} , 10^{-10} , 10^{-12}). They were subjected to a 24-hour incubation period at 37 °C. MRS agar was used for the formulation containing *L. plantarum*, and MacConkey agar for *E. coli*. The colony count (CFU/mL) was calculated using the formula as follows,

$$\text{CFU/mL} = (\text{No. of colonies} \times \text{Dilution factor}) / (\text{Volume of culture plated (mL)})$$

Statistical Analysis

Data from each experiment was reported as mean $\pm$ standard error mean (SEM). The statistical significance was determined using Graph pad prism software (version 6.0, USA) to assess the statistical significance of the data by one-way analysis of

variance (ANOVA) followed by Tukey's multiple comparison test. The control group was compared with the other groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: non-Significant.

Results**Standardization of *L. Plantarum***

After incubation, the *L. plantarum* colonies demonstrated a creamy white opaque colour. The appeared colonies were small, round, and exhibited a raised elevation with an entire margin.

Catalase Test and Oxidase Test

The catalase test was negative, as no bubbles were observed. *L. plantarum* restricts the breakdown of H_2O_2 and further leads to the accumulation of H_2O_2 within the bacterial cell, which can limit the proliferation of pathogenic micro-organisms. In the case of the oxidase test, no change was observed on the oxidase disc within 15-30 seconds after adding an isolated bacterial colony, indicating negative oxidase test results.

Triple Sugar Iron tTest

The *L. plantarum* TSI test results showed an alkaline butt (red) and an acid slant (yellow), with no gas or H_2S production. *L. plantarum* can aerobically ferment lactose and sucrose to generate acid, as evidenced by the yellow slant.

Table 1: The biochemical reactions for the diagnostic features of *L. plantarum* using Vitek 2C identification

Biochemical Test	Reaction*
dGAL (D-Galactose)	-
LeuA (Leucine Aminopeptidase)	+
ELLM (L-Ellman)	-
PheA (Phenylalanine Aminopeptidase)	+
ProA (Proline Aminopeptidase)	+
PyrA (Pyrrolidonyl Aminopeptidase)	-
dCEL (D-Cellobiose)	+
TyrA (Tyrosine Aminopeptidase)	+
APPA (L-Alanine Aminopeptidase)	-
dGLU (D-Glucose)	+
dMNE (D-Mannitol)	+
dMAL (D-Maltose)	+
SAC (Sucrose)	+
ARB (L-Arabitol)	+
NAG (N-Acetyl-Glucosamine)	+
BGLUi (Beta-Glucosidase)	+

URE (Urease)	-
BGURi (Beta-Glucuronidase)	-
BGALi (Beta-Galactosidase)	-
AARA (Alpha-Arabinosidase)	-
AGALi (Alpha-Galactosidase)	-
BMAN (Beta-Mannosidase)	-
ARG (Arginine Dihydrolase)	-
PVATE (Phosphatidylinositol Phospholipase C)	-
MTE (Alpha-Mannosidase)	+
ESC (Esculin Hydrolysis)	+
BdFUC (Beta-D-Fucosidase)	-
BNAGi (Beta-N-Acetylglucosaminidase)	-
AMANi (Alpha-Mannosidase)	-
AIFUC (Alpha-L-Fucosidase)	-
PHOS (Phosphatase)	-
IARA (Inositol)	-
dRIB2 (D-Ribose)	+
OPS (Ornithine Decarboxylase)	-
AARAF (Alpha-L-Arabinofuranosidase)	-
dXYL (D-Xylose)	-
GRAM (Gram Stain)	+
MORPH (Morphology)	-
AERO (Aerobic Growth)	?

* (+) Positive enzyme activity / Positive fermentation, (-) No activity / No fermentation, (?) Uncertain

VITEK Analysis

The biochemical makeup of *L. plantarum* was examined using the VITEK 2C identification technology.⁵⁴ *L. plantarum* provided several satisfactory findings, which are essential for species identification. Aminopeptidases, including leucine, proline, and tyrosine, verified *L. plantarum*'s distinctive enzymatic profile (Table 1). The outcomes additionally displayed beta-glucoside activity and alpha-mannosidase activity.

Characterization of Okara

Pharmacognostic Evaluations

The pharmacognostic characteristics of okara revealed a total ash value of $4 \pm 0.02\%$, an acid-insoluble value of $1 \pm 0.01\%$, and a water-soluble ash value of 3.75% which was within the acceptable range and is not more than the standard value, i.e., 5% .

Bradford Assay of Okara

The present investigation utilized the Bradford test to assess okara's protein content quantitatively.

BSA was utilized as a standard. The BSA standard curve was obtained with $R^2 = 0.9948$ and the equation ($y = 0.0006x + 1.1384$). The range of protein concentrations in okara was $52.25 \mu\text{g/mL}$ to $1042.25 \mu\text{g/mL}$, where higher absorbance values corresponded to more significant protein concentrations.

Prebiotic Efficacy of Okara

Growth Kinetics of Okara

The growth kinetics were investigated using resubstituted MRS broth with okara at various concentrations (1%, 2%, 3%, and 4%), *L. plantarum* as a probiotic, and FOS as a comparison sugar source. The okara exhibited robust growth at 2% of okara concentration with the specific growth rate (μ) at 0.064 at 2 h, and it increased up to 1.73 at 48 h, demonstrating significant and sustained growth (Table 2).

pH and % Titratable Acidity

L. plantarum can produce organic acids by fermentation of sugars, and a majority of lactic

acid is produced. Initially, it was neutral to partially alkaline. The amino acid composition of okara can contribute to an increase in pH. Among all the okara concentrations, 2% was the most

effective at promoting bacterial growth and lactic acid production. Although 3% okara produced a significant pH reduction, 1% and 4% concentrations had less influence on pH (Figure 1b).

Table 2: Specific Growth Rate of *L. plantarum* in response to varying concentrations of okara as a prebiotic over different time intervals

Time (hr)	1%	2%	3%	4%	FOS	Control
μ_2	0.044	0.064	0.096	0.075	0.068	0.0175
μ_4	0.064	0.100	0.148	0.1625	0.149	0.0295
μ_6	0.084	0.323	0.177	0.211	0.517	0.103
μ_{24}	0.546	0.662	0.866	0.755	1.225	0.478
μ_{48}	0.918	1.737	1.209	1.016	1.896	1.245
μ_{avg}	0.3312	0.5772	0.4992	0.4439	0.771	0.3746

The % titratable acidity for *L. plantarum* gradually increased with time and various concentrations of okara. At 0 h, acidity levels were relatively low across all concentrations. Acidity gradually increased during incubation. By 48 hours, the bacteria

had optimum acidity levels of 2% (0.94) and 3% (0.87), respectively, with FOS having the highest acidity at 1.10 and the control at 0.92 representing *L. plantarum*'s highest metabolic activity and acid production (Figure 1c).

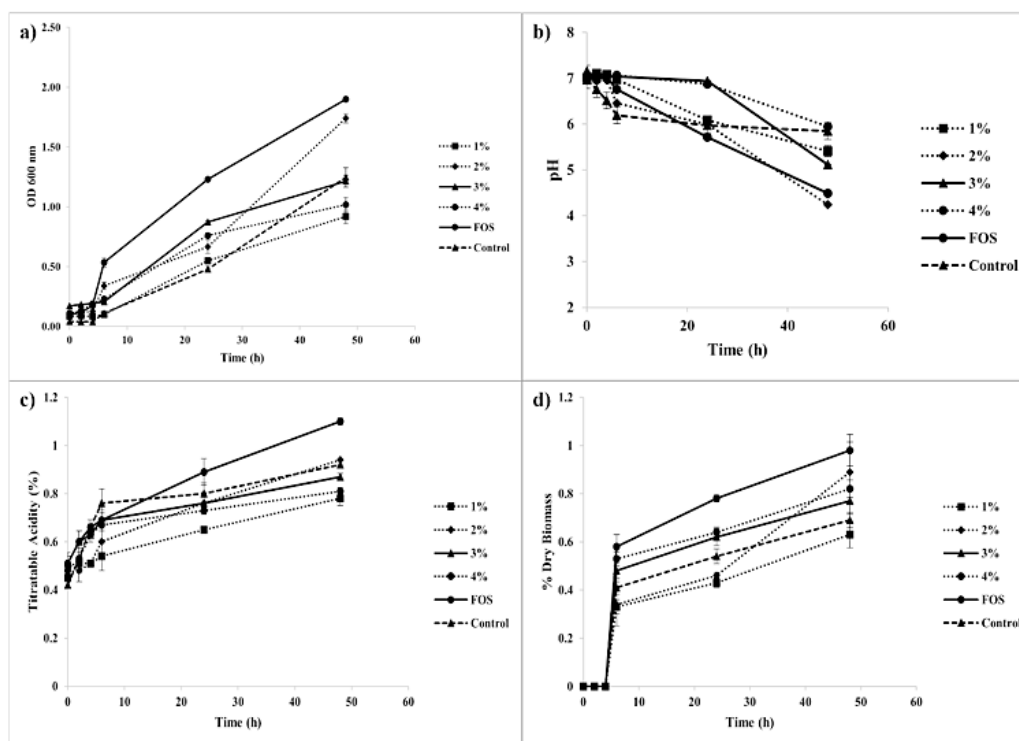


Fig.1: *L. plantarum* with okara (1%, 2%, 3%, and 4%), FOS, and Control (without prebiotic) for a) Effect on the growth of *L. plantarum*, b) Effect on pH of *L. plantarum*, c) Effect on % titratable acidity of *L. plantarum* with okara, d) % Dry biomass of *L. plantarum* at different time intervals

Dry Biomass

Dry biomass concentrations (g/L) of *L. plantarum* were determined in various concentrations of okara, fructooligosaccharides (FOS), and control conditions periodically. All concentrations had no dry biomass at 0, 2, and 4 hours. As described in Figure 1d, at 6 hours, biomass started to accumulate. Notably, 2%

okara and FOS produced greater biomass than the other concentrations, with values of 0.34 and 0.58 g/L, respectively. In contrast, the remaining okara concentrations (1%, 3%, and 4%) and the control had lower biomass concentrations throughout the observation period, indicating slower biomass accumulation.

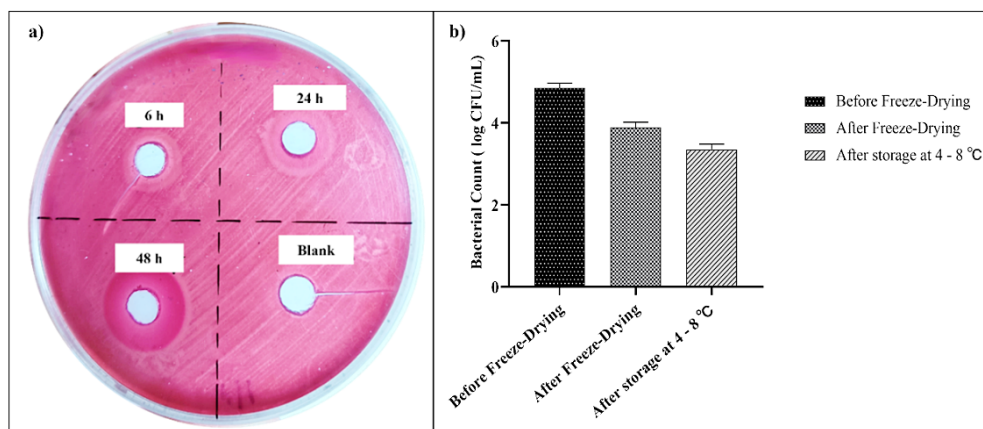


Fig.2. a) Anti-microbial activity against *E. coli* at 6 h, 24 h, and 48 h of exposure, b) Viability enumeration of *L. plantarum* before freeze-drying, after freeze-drying and after storage at 4-8 °C

Anti-Microbial Activity

The zone of inhibition (ZOI) increased significantly for all *Lactobacillus* samples between 6 and 48 h, indicating a time-dependent effect (Figure 2a). After 6 h, the 7.1 ± 0.3 mm ZOI showed significant inhibition. The ZOI rose after 24 h by 9.2 ± 0.3 mm, indicating greater susceptibility to *E. coli*. The ZOI significantly increased after 48 h up to 16.9 ± 0.2 mm, demonstrating that *E. coli* is responsive to *Lactobacillus*' anti-microbial effect.

Evaluation of Freeze-Dried Powder

A novel formulation of *L. plantarum* and okara has been developed as a freeze-dried powder. With the incorporation of sugars like mannitol, sorbitol, and maltodextrin. 9 batches were developed by adding okara, mannitol, sorbitol, and maltodextrin with probiotics with different ratios. The 3 best batches with the highest yields were selected for further evaluation from the nine batches.

The three scrutinized batches (B1, B2, and B4) were examined for yield, flow properties, and particle size metrics to determine the most optimal batch after lyophilization of freeze-dried powder. Batch B1 yielded 85.2% with a 2:1 okara-mannitol ratio, Batch

B2 yielded 84.6% with the same ratio, and Batch B4 yielded the highest yield at 86.6%. While Batch B4 had the highest yield due to the okara: sorbitol ratio (2:1), Batch B2 was distinguished by its good flow and particle size characteristics. Batch B2 exhibits a less significant drying loss (2.1%) than Batch B4 (8.6%), indicating higher stability and reduced moisture retention. This is essential to maintaining powder quality throughout the process. Batch B2 exhibited less agglomeration than Batch B1 (34.9°) and Batch B4 (30.9°), with the lowest angle of repose (26.5°) indicating better flow.

B1 exhibited larger particle sizes than Batch B2 (87.7 μm and 82.03 μm , respectively) (Table 3). The arithmetic mean for Batch B4 is 85.15 μm , and the geometric mean was 82.53 μm . Smaller particle sizes increase flowability and reduce agglomeration. Furthermore, laser diffraction (Malvern Mastersizer 2000) was used to determine the particle size of batch B2. A cumulative undersize curve was created, enabling the identification of granule sizes at the 10th, 50th, 90th, and 100th percentiles (D10, D50, D90, and D100). The estimation covered a range of 4.537 μm to 434.390 μm .

Table 3: Composition of the powder formulation, and micromeritic properties of the powder formulation

Batch	Okara (g)	Mann-itol (g)	Sorbi- tol (g)	Maltode -xtrin (g)	<i>L.plantarum</i> (CFU/mL)	Powder Yield	Drying Loss on (%)	Bulk Density (%)	Tapped Density	Angle of Repose	Hausner Ratio	Carr's Index	Arithmetic Mean	Geometric Mean
B1	2	1	-	-	1.2×10^9	85.2	3.4±0.05	0.11	0.16±0.04	34.9±0.02	1.52±0.07	52.94±0.01	91.04	89.67
B2	2	2	-	-	1.2×10^9	84.6	2.1±0.03	0.13±0.03	0.19±0.01	26.5±0.04	1.5±0.06	45±0.04	87.7	82.03
B3	2	4	-	-	1.2×10^9	83.9	7±0.03	0.17±0.01	0.23±0.03	26.5±0.03	1.33±0.03	33.33±0.03	98.25	82.93
B4	2	-	1	-	1.2×10^9	86.6	8.6±0.02	0.12±0.04	0.17±0.06	30.9±0.03	1.35±0.04	45.98±0.06	85.15	82.53
B5	2	-	2	-	1.2×10^9	84	4.7±0.01	0.17±0.03	0.22±0.01	32.6±0.09	1.31±0.03	30.07±0.03	85.15	84.24
B6	2	-	4	-	1.2×10^9	83.3	8.9±0.05	0.26±0.03	0.27±0.02	33.42±0.03	1.05±0.02	8.2±0.01	98.25	85.65
B7	2	-	-	1	1.2×10^9	79.6	2.7±0.04	0.12±0.02	0.16±0.03	34.21±0.02	1.31±0.06	22.55±0.03	78.6	81.65
B8	2	-	-	2	1.2×10^9	82.5	3.9±0.01	0.14±0.01	0.21±0.05	32.21±0.07	1.47±0.08	39.1±0.08	85.15	80.98
B9	2	-	-	4	1.2×10^9	80	3±0.03	0.2±0.03	0.27±0.01	31.38±0.01	1.35±0.03	39.29±0.07	95.23	93.12

*Values are expressed as Mean ± SD (n=3)

Evaluation of Reconstituted Freeze-Dried Powder

Viability Enumeration

The objective of the study was to determine the vitality of bacterial cells during various stages of preservation, particularly before and after freeze-drying, as well as after storage at 4 - 8 °C. Before freeze-drying, the initial bacterial count was 4.06 ± 0.023 log CFU/mL (or 4.66 ± 0.023 log CFU/g). The bacterial count decreased to 3.78 ± 0.045 log CFU/mL (or 4.40 ± 0.045 log CFU/g) after the freeze-drying procedure, suggesting a slight reduction in viability. A bacterial count of 3.54 ± 0.015 log CFU/mL (or 4.16 ± 0.015 log CFU/g) was obtained after additional storage at 4 - 8 °C for 7 days, indicating a progressive decrease in viability over the storage time (Figure 2b).

In-vitro Efficacy by Co-Culture Technique

A mixed culture technique was used to study the effect of synbiotic freeze-dried powder formulation on *E. coli*. The formulation with probiotic and prebiotic was incubated alone in MRS media to observe the growth of *L. plantarum* and in the presence of *E. coli* culture to study the mixed culture effect. When the formulation was incubated in media without any pathogen, there was a sustained increase in *L. plantarum* at 24 h (8.26 ± 0.12 log CFU/mL) and 48 h (9.68 ± 0.19 log CFU/mL). The bacterial colony count at 0 h was found to be 5.96 ± 0.06 CFU/mL for group 1 (control of *E. coli*), which increased to 7.22 ± 0.09 CFU/mL at 24 h and 8.20 ± 0.05 CFU/mL at 24 h. Group 2 (Formulation + *E. coli*) showed 5.37 ± 0.08 CFU/mL initially, while at 24 h, the colony count increased to 6.31 ± 0.05 CFU/mL and 7.25 ± 0.09 CFU/mL at 48 h. For Group 3 (Control of *L. plantarum*), the colony count observed was 6.06 ± 0.13 CFU/mL at 0 h, gradually increasing to 7.76 ± 0.27 at 24 h and 8.64 ± 0.21 at 48 h. In group 4, containing only formulation, the colony count was observed as 5.98 ± 0.02 CFU/mL at 0 h, 8.26 ± 0.12 CFU/mL at 24 h and 9.68 ± 0.19 CFU/mL at 48 h (Figure 3b).

Discussion

The staining observed thick peptidoglycan layers⁴⁵ and the presence of gram-positive bacilli with the characteristic *L. plantarum* staining appearance, which showed purple-coloured, rod-shaped bacteria with single rods, pairs, and short chains with apparent length variations.

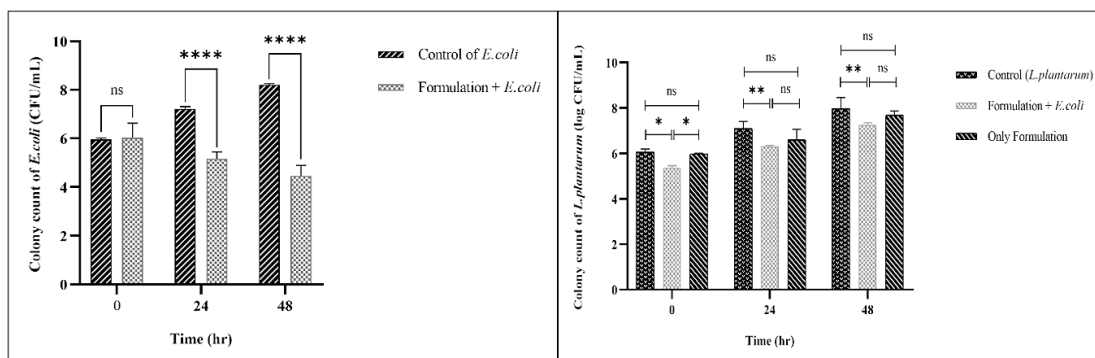


Fig.3. Effect of freeze-dried formulation on the count of a) *E.coli* and b) Co-culture of *L.plantarum* with *E.coli*.

Data were expressed as mean $\pm$ SEM, $n=3$ and Tukey's Multiple comparisons analysis by two-way analysis of variance (ANOVA). The control group is compared with the other groups.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns: non-significant.

The catalase enzyme neutralized the bactericidal effects of hydrogen peroxide (H_2O_2). It promotes the breakdown of H_2O_2 into water and oxygen and also inhibits the antibacterial properties of hydrogen peroxide, and its quantity in bacteria has been associated with pathogenicity.^{55–57} In case of oxidase test, *L. plantarum* lacks cytochrome c oxidase. This absence enabled the *L. plantarum* to survive in anaerobic or low-oxygen conditions with the ability to produce acidic conditions that inhibit the proliferation of pathogenic micro-organisms. The crimson buttocks indicated that the *L. plantarum* fermented glucose anaerobically in TSI. The absence of blackening indicated the absence of H_2S generation, whereas the absence of bubbles confirms the absence of gas production.

In VITEK analysis the bacterium can be recognized easily because of its esculin hydrolysis, absence of urease production, and negative reactivity to other enzyme activities such as beta-glucuronidase, beta-galactosidase, and arginine dihydrolase. These tests are biochemical indicators that distinguish the *L. plantarum* from the other *Lactobacillus* species. The *L. plantarum* profile showed various types of carbohydrates, including D-cellobiose, D-glucose, D-mannitol, D-maltose, sucrose, L-arabitol, N-acetylglucosamine, and D-ribose. These demonstrate the fermentation ability of *L. plantarum*. All the biochemical characteristics confirmed the presence of *L. plantarum* precisely.

The pharmacognostic tests of okara yielded positive confirmatory tests such as the molish test for carbohydrates, the biuret test for proteins, and the bromine water test for tannins. In the case of the growth kinetics of okara, the doubling time (g) for 2% was 0.39 h at 48 h. Similarly, FOS exhibited a consistent, substantial proliferation with a specific growth rate of 1.89 at 48 h and doubling time at 0.36 h, confirming its ability to promote bacterial growth. In contrast, the control exhibited gradual development with an initial specific growth rate of 0.017 at 2 h and increased to 1.24 after 48 h (Figure 1a). The doubling time for control was 0.55 h at 48 h, indicating that the *L. plantarum* growth was substantially less supported without adding okara. Other okara concentrations showed a relatively small increase, with an initial specific growth rate of 0.044 at 2 h and 0.91 at 48 h, corresponding doubling time of 0.75. The 3% okara concentration proliferated faster than 1% but less effectively than 2%. Additionally, 4% okara showed noteworthy growth, with an initial specific growth rate of 1.016 at 48 h, with a doubling time of 0.68 h at 48 h. A significant increase was provided by 2% okara and FOS, which indicated more excellent specific growth rates and shorter doubling time, indicating its efficacy as a prebiotic.

The pH declined substantially from 7 to 4.24 after 48 h, indicating significant fermentation activity and optimum conditions for *L. plantarum*. Correspondingly, FOS presented a considerable pH

decrease, representing that the bacteria produced acid rapidly. In contrast, the control group (without prebiotics) had the minimum variation in pH, initially at 6.9 and declining gradually to 5.8 at 48 h. This emphasizes the role of okara as a prebiotic and FOS in *L. plantarum*'s growth and metabolic activity.

Although Batch B4 has the shortest arithmetic mean particle size, Batch B2's geometric mean particle size is also reasonably small, indicating strong flow properties without compromising stability. While Batch B1 had the highest output, its flow properties are less favourable, with a Carr's Index of 52.94 and a higher angle of repose, making it more prone to handling difficulties. Despite having a higher yield and better particle size distribution, Batch B4 had a higher drying loss and less favourable flow properties than Batch B2. Batch B2 was selected for its balanced combination of low drying loss, good flow properties, and relatively small particle size.

Viability Enumeration findings implied that although bacterial viability is affected by freeze-drying and subsequent cold storage, the effects were minor. Consequently, freeze-drying and storage at 4 - 8 °C for 7 days can be considered an effective method to preserve bacteria alive, even if there will always be some bacterial count reduction.

In-vitro efficacy by co-culture technique showed steady growth at 24 h and 48 h control with *E. coli* (Figure 3a). When the formulation was incubated in *E. coli* culture, there was an increase in *L. plantarum* growth and a decrease in the growth of *E. coli*. This inferred that *E. coli* does not inhibit *L. plantarum*, whereas prebiotics support the development of *L. plantarum*.

Conclusion

The current research successfully developed a synbiotic nutraceutical formulation containing *L. plantarum* as a probiotic and okara as a prebiotic, using freeze-drying process. Batch B2 emerged as the most preferable among the nine selected batches because of its optimal properties. Okara demonstrated the potential prebiotic efficacy through growth kinetics of *L. plantarum*, by significantly enhancing its growth rate and biomass accumulation. The viability studies confirmed that the freeze-drying process effectively upheld the viability

of bacterial cells, with minimal reduction in bacterial count after storage. The formulation's *in-vitro* efficacy showed substantial growth of *L. plantarum* and inhibition of *E. coli*, indicating the prebiotic's role in promoting probiotic growth and suppressing pathogens. In conclusion, the study highlights the successful development and optimization of a synbiotic nutraceutical formulation that combines the benefits of probiotics and prebiotics, offering significant potential for enhancing gut health and contributing to improved nutrition.

Future studies will involve *in-vivo* animal trials to assess the efficiency of soybean-based synbiotic powder containing *L. plantarum* in enhancing gut health and altering gut microbiota. Long-term investigations could determine its preventative and therapeutic efficacy against gastrointestinal diseases. Furthermore, clinical trials could test its application in humans, ensuring scalability and application effectiveness.

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Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

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Not Applicable.

Author Contributions

- **Roshani Rajendra Pagar:** Writing - original draft, Visualization, Software, Methodology,

Investigation, Formal analysis, Data curation.

- **Sanjeevani Deshkar:** Conceptualization, Design of study, Revising and approval of manuscript, Validation, Supervision, and Data curation.
- **Rewati Choudhary:** Methodology, Investigation, Formal analysis.
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