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***In- vitro* evaluation of Indian indigenous green leafy vegetables for prebiotic potential in synbiotic yoghurt formulation**

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Abstract

The study aimed to explore the effect of incorporation of green leafy vegetables on the growth of bioyoghurt cultures for the development of synbiotic yoghurt by harnessing the prebiotic potential of Indian green leafy vegetables in combination with probiotics. Indian indigenous green leafy vegetables have long been integral to traditional diets, offering a rich source of vitamins, minerals, and bioactive compounds. This study investigates the *in-vitro* prebiotic potential of five such green leafy vegetables; *Spinacia oleracea* (spinach), *Raphanus sativus* (raddish leaves), *Coriandrum sativum* (coriander leaves), *Murraya koenigii* (curry leaves) and *Mentha spicata* (mint leaves) within synbiotic yoghurt formulations. The study evaluated the impact of these green leafy vegetables on the growth and viability of yoghurt culture and probiotic strains *Lactobacillus acidophilus* LA-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12. Results indicated that curry leaves demonstrated the strongest prebiotic potential, significantly enhancing probiotic viability and accelerating yoghurt fermentation time, especially in combination with the probiotic strains. Curry leaves also showed high prebiotic activity scores (PAS) and prebiotic indices (PI) for *Lactobacillus acidophilus* LA-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12, comparable to the standard prebiotic inulin. Further research could focus on optimizing the concentrations and combinations of these green leafy vegetables to enhance their prebiotic efficacy, as well as exploring their applications in other probiotic-rich foods beyond yoghurt.

Keywords: Prebiotics, polyphenols, green leafy vegetables, leaf extract, yoghurt

Introduction

The utilization of Indian indigenous green leafy vegetables in food products has a longstanding history due to their rich nutritional content and potential health benefits [1-3]. Green leafy vegetables like spinach, radish leaves, curry leaves, mint, and coriander are staples in traditional Indian diets, offering a wide array of vitamins (A, C, and K), minerals (iron, calcium, magnesium), and essential bioactive compounds like polyphenols and flavonoids [4]. In recent years, these green leafy vegetables have attracted attention for their prebiotic potential, that can stimulate the growth of beneficial gut bacteria [5-12]. In the context of prebiotic potential, these green leafy vegetables are rich in dietary fibers, polyphenols and non-digestible oligosaccharides. They stimulate the growth of probiotics in the gut by serving as food for beneficial bacteria [13-17]. Spinach and radish leaves which are high in vitamin A, B C, E, K contain dietary fibers, polyphenols and flavonoids that can enhance the fermentation activity of gut bacteria, increasing short-chain fatty acid production, which is essential for colon health and also helps in reduction of oxidative stress and inflammation [18]. Polyphenols and fibers, such as inulin and oligosaccharides, found in curry leaves, serve as prebiotics, selectively stimulating the growth of probiotic bacteria like *Lactobacillus* and *Bifidobacteria*. It has been shown to improve digestion and act as a mild laxative, helping to regulate gut microbiota [19, 20]. The high antioxidant content in mint and coriander helps neutralize free radicals, reducing oxidative damage at the cellular level. This effect is beneficial in preventing chronic conditions like heart disease and cancer. The anti-inflammatory properties of these plants can also alleviate gastrointestinal distress and protect against ulcers [21]. Incorporating these prebiotic-rich green leafy vegetables in synbiotic product formulations can enhance the health benefits of the product.

However, while these green leafy vegetables offer numerous health advantages, it is also essential to consider their potential negative health impacts when incorporated into functional food products. Spinach and radish leaves contain oxalates, which can contribute to kidney stone

formation in susceptible individuals when consumed in large quantities [22]. Additionally, some green leafy vegetables, like coriander, may trigger allergic reactions in sensitive individuals, leading to gastrointestinal distress or skin irritation [23]. Radish leaves are known to contain goitrogens, compounds that interfere with thyroid function by inhibiting iodine uptake. This could be a concern for individuals with thyroid issues if these green leafy vegetables are consumed excessively [24]. Therefore, it is important to carefully consider these potential side effects when incorporating such green leafy vegetables into functional and synbiotic products.

Synbiotic products are designed to deliver both probiotics (beneficial bacteria such as *Lactobacillus* and *Bifidobacteria*) and prebiotics (non-digestible food components that promote the growth of probiotics), offering a powerful combination for improving gut health. The synergistic effect of probiotics and prebiotics improves gut microbiota balance, aids digestion, and enhances nutrient absorption [25, 26]. Incorporating green leafy vegetables into synbiotic yoghurt formulations offers a promising approach to develop functional foods that promote gut health. The high fiber content and polyphenol content of green leafy vegetables provides an ideal substrate for the fermentation of probiotics, increasing their effectiveness [27, 28]. Furthermore, these green leafy vegetables bring additional micronutrients to the yoghurt, enriching it with iron, calcium, and vitamins, making the product more nutritionally appealing [29]. Antimicrobial properties of mint and curry leaves further help preserve the yoghurt by inhibiting the growth of spoilage microorganisms, enhancing both its safety and shelf life, offering an alternative to synthetic preservatives [30, 31]. By combining the probiotic benefits of yoghurt with the prebiotic potential of these green leafy vegetables, synbiotic formulations can improve digestive health, boost immunity, and provide a nutrient-dense food option for health-conscious consumers. This study aims to investigate the *in-vitro* prebiotic potential of five Indian indigenous green leafy vegetables- Spinach, radish leaves, curry leaves, mint, and coriander, in relation to synbiotic yoghurt formulations

Materials and Methods

Bacterial cultures

Yoghurt cultures *Streptococcus thermophilus*, *Lactobacillus delbrückii* ssp *bulgaricus* (STLB) and Probiotic cultures *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 were obtained from the Department of Dairy Microbiology, Dairy Science College, Hebbal, Bengaluru -24. DVS cultures maintained in Yeast Glucose Chalk Litmus Milk (YGCLM) as stock were used and the working cultures were maintained in reconstituted sterile skim milk. For prebiotic activity evaluation *L. acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 were propagated in a De Man Rogosa Sharpe (MRS) (HiMedia) broth, at 37 °C, in anaerobic conditions, for atleast 48 h and *Escherichia coli* was taken as a representative enteric bacteria and was propagated aerobically in nutrient broth (Hi-Media) at 37 °C, for 24 h.

Commercial prebiotics

Inulin (Nice chemicals) was used as the reference prebiotic for calculating prebiotic activity score and prebiotic index.

Collection of plant materials

Fresh spinach (*Spinacia oleracea*), raddish leaves (*Raphanus sativus*), coriander leaves (*Coriandrum sativum*), curry leaves (*Murraya koenigii*) and mint leaves (*Mentha spicata*) were collected from local markets of Hebbal and the leaves were separated from leaf stalks and cleaned to remove all foreign matter adhered using potable water.

Preparation of juice extracts and powder of green leafy vegetables

Juice extracts of green leafy vegetables at different

concentrations such as 10% (10 g sample in 90mL distilled water), 25% (25 g sample in 75 ml distilled water) and 50% (50 g sample in 50mL distilled water) were prepared using mortar and pestle and filtered using a muslin cloth. The extracts were heat treated at 85 °C for 30 min to eliminate microbial contamination. For prebiotic activity assay and viability assessment, the cleaned leaves were oven dried at 55 °C for 16-18 h and the dried leaves were ground into fine powder in a mixer and stored in air tightened containers at 4°C [10].

Effect of juice extracts on growth dynamics of yoghurt and probiotic bacterial cultures

Assessment of fermentation time of milk, direct microscopic count (DMC) and titratable acidity

Yoghurt cultures *Streptococcus thermophilus*, *Lactobacillus delbrückii* ssp *bulgaricus* and probiotic *Lactobacillus acidophilus*-LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 were activated in reconstituted skim milk (RSM) at 37 ± 2 °C or 24 h. Sterilized 9% RSM was used as a basal growth medium. Different concentrations (10, 25 and 50%) of juice extract of green leafy vegetables sterilized by heating at 85°C for 30 min was incorporated into RSM at 1% level. The activated yoghurt culture (2%), probiotic culture (5% each) and their combination was inoculated into basal growth media along with juice extracts and incubated at 42 ± 2 °C. Fermentation time, DMC and titratable acidity of curd formed were evaluated by standard procedure [32, 33].

Assessment of viability by spread plate method

The viability of yoghurt and probiotic cultures was assessed using the spread plate method on M17, MRS and *Bifidobacterium* agar supplemented with 5% green leafy vegetable powder (GLVP). The media, along with the GLVP, was sterilized by autoclaving. After cooling, 0.1 mL of activated yoghurt and probiotic cultures serially diluted up to 10⁻⁷ dilution using sterile phosphate buffer, were inoculated onto the surface of the agar plates with GLVP and spread evenly using the spread plate technique. The plates were then incubated at 37°C under anaerobic conditions for 24 - 48 h to allow for bacterial growth. Agar without GLVP served as the control to compare the growth dynamics. The results were analyzed based on colony counts and total viable cell count (log₁₀ CFU/mL) was calculated, indicating the effect of GLVP on the viability of the yoghurt and probiotic cultures [34].

Evaluation of prebiotic potential using prebiotic activity score (PAS) and prebiotic index (PI)

The experiment was carried out following the method described by [35] with some modifications. MRS broth and MRS broth with 1% (w/v) of GLVP was inoculated with 1% (v/v) of an overnight culture of each of the probiotic strains. Cultures were incubated at 37 °C under anaerobic conditions. *Escherichia coli* was included in the assay as the enteric organism to compare the selective stimulation of beneficial bacteria. Overnight culture of *E. coli* was inoculated at 1% (v/v) in nutrient broth and nutrient broth with 1% (w/v) of GLVP and incubated at 37 °C under aerobic conditions. At 0 and 24 h of incubation, bacterial counts (CFU/mL) of each of the inoculums were enumerated by plating on MRS agar for probiotic strains and endo agar for *E. coli*. The plates were incubated at 37 °C for 24 - 48 h anaerobically and aerobically respectively. Commercial inulin was included as a positive control and broth without any supplement acted as the negative control. Each assay was performed in triplicate.

Prebiotic activity score

The prebiotic activity score (PAS) measures the ability of a substrate to selectively enhance the growth of probiotic bacteria (like *Lactobacillus* and *Bifidobacterium*) relative to non-beneficial bacteria. The equation for the Prebiotic Activity Score (PAS) as described by [36] is:

$$PAS = \frac{(\log \text{CFU/mL of probiotics at 24h on prebiotic} - \log \text{CFU/mL of probiotics at 0h on prebiotic})}{(\log \text{CFU/mL of probiotics at 24h on glucose} - \log \text{CFU/mL of probiotics at 0h on glucose})}$$

$$\frac{(\log \text{CFU/mL of enteric bacteria at 24h on prebiotic} - \log \text{CFU/mL of enteric bacteria at 0h on prebiotic})}{(\log \text{CFU/mL of enteric bacteria at 24h on glucose} - \log \text{CFU/mL of enteric bacteria at 0h on glucose})}$$

This equation compares the relative growth of probiotics and enteric bacteria (such as *E. coli*) on a prebiotic substrate versus glucose. A positive PAS score indicates favorable growth of probiotics and minimal growth of enteric bacteria.

Prebiotic index (PI)

The Prebiotic Index (PI) is a quantitative measure used to evaluate the beneficial effects of various substrates (such as prebiotics) on the growth of probiotic bacteria. PI was calculated using the equation previously described by Elbermawi *et al.* [37] and Dawood *et al.* [38]. The basic formula for PI is used to compare the growth of probiotics in a prebiotic-supplemented medium to their growth in a control medium (glucose).

$$PI = \frac{\text{CFU of probiotics in GLVP supplemented medium}}{\text{CFU of probiotics in control medium}}$$

A PI greater than 1 suggests that the prebiotic has a beneficial effect on the probiotic strain, promoting its growth. Conversely, a PI close to 1 indicates that the tested substrate has low efficacy in enhancing probiotic growth.

To account for the dynamic changes in bacterial populations, the basic PI can be expanded by incorporating the specific growth rate of different bacterial groups [39]. The logarithmic equation governing bacterial growth can be expressed as:

$$\ln N_t = \ln N_0 + mt$$

Where: N_t is the bacterial population after a time t , N_0 is the initial bacterial population and m is the specific growth rate

(h^{-1}).

In the exponential growth phase, the specific growth rate reaches a maximum ($m_{\max}$) for each bacterial group. The modified prebiotic index (PI_m) incorporates these maximum growth rates across different bacterial populations as:

$$PI_m = m_{\max} \text{Lactobacillus} + m_{\max} \text{Bifidobacterium} - m_{\max} \text{E.coli}$$

An increase in the maximum growth rates of beneficial bacteria such as lactobacilli and bifidobacteria, contributes positively to the PI_m , while an increase in the harmful bacterial groups like *E. coli*, negatively impacts the index. Therefore, the modified prebiotic index provides a more detailed analysis of the impact of prebiotics, considering both the beneficial and harmful bacterial populations in the gut [40].

Statistical analysis

The data obtained were analyzed using R studio (version 4.2.2). Data was expressed as means $\pm$ standard deviation. Differences among means were analyzed using analysis of variance (ANOVA) in the agricolae package. Significant differences between treatments were evaluated using Tukey's HSD test at $P=0.05$.

Results and Discussion

Assessment of fermentation time of milk, direct microscopic count (DMC) and titratable acidity

The fermentation time of milk in presence of green leafy vegetable extracts is presented in Table 1.

Table 1: Fermentation time of milk in presence of green leafy vegetable extracts

Green leafy vegetables	Concent-ration (%)	Fermentation time (h)			
		STLB (2%)	LA-5 (5%)	BB-12 (5%)	STLB+LA-5+BB-12 (2%+2.5%+2.5%)
Control (No extract)	0	4.6 $\pm$ 0.57 ^{ab}	23.3 $\pm$ 1.1 ^{ab}	47.0 $\pm$ 1.7 ^a	4.5 $\pm$ 0.43 ^a
Spinach	10	4.5 $\pm$ 0.50 ^{ab}	23.8 $\pm$ 0.28 ^a	45.6 $\pm$ 4.04 ^a	4.1 $\pm$ 0.10 ^{ab}
	25	4.7 $\pm$ 0.68 ^{ab}	23.0 $\pm$ 1.0 ^{abc}	46.0 $\pm$ 3.46 ^a	4.1 $\pm$ 0.15 ^{ab}
	50	4.6 $\pm$ 0.65 ^{ab}	18.2 $\pm$ 0.28 ^{de}	47.0 $\pm$ 1.73 ^a	4.1 $\pm$ 0.10 ^{ab}
Radish leaves	10	5.0 $\pm$ 1.0 ^a	23.1 $\pm$ 0.76 ^{ab}	45.3 $\pm$ 4.61 ^a	4.5 $\pm$ 0.43 ^a
	25	4.8 $\pm$ 0.80 ^{ab}	23.5 $\pm$ 0.86 ^{ab}	47.3 $\pm$ 1.15 ^a	4.3 $\pm$ 0.26 ^{ab}
	50	4.7 $\pm$ 0.68 ^{ab}	18.6 $\pm$ 0.57 ^d	48.2 $\pm$ 0.28 ^a	4.2 $\pm$ 0.20 ^{ab}
Coriander leaves	10	4.7 $\pm$ 0.26 ^{ab}	23.8 $\pm$ 0.28 ^a	47.0 $\pm$ 1.73 ^a	4.2 $\pm$ 0.25 ^{ab}
	25	4.8 $\pm$ 0.1 ^{ab}	23.8 $\pm$ 0.76 ^a	47.3 $\pm$ 1.15 ^a	4.4 $\pm$ 0.36 ^{ab}
	50	4.1 $\pm$ 0.05 ^{ab}	18.0 $\pm$ 2 ^{de}	45.3 $\pm$ 4.61 ^a	4.1 $\pm$ 0.15 ^{ab}
Curry leaves	10	4.8 $\pm$ 0.46 ^{ab}	22.8 $\pm$ 1.04 ^{abc}	43.6 $\pm$ 5.13 ^a	3.3 $\pm$ 0.20 ^{cd}
	25	3.8 $\pm$ 0.32 ^{ab}	20.6 $\pm$ 1.15 ^{bcd}	41.0 $\pm$ 9.64 ^a	3.2 $\pm$ 0.25 ^{cd}
	50	3.1 $\pm$ 0.28 ^b	17.3 $\pm$ 0.57 ^e	28.7 $\pm$ 1.52 ^b	2.8 $\pm$ 0.30 ^d
Mint leaves	10	4.3 $\pm$ 0.46 ^{ab}	23.8 $\pm$ 0.28 ^a	48.0 $\pm$ 1 ^a	4.3 $\pm$ 0.32 ^{ab}
	25	4.2 $\pm$ 0.28 ^{ab}	23.3 $\pm$ 1.15 ^{ab}	47.7 $\pm$ 1.52 ^a	4.2 $\pm$ 0.15 ^{ab}
	50	4.2 $\pm$ 0.28 ^{ab}	20.0 $\pm$ 2 ^{cde}	47.3 $\pm$ 1.15 ^a	4.1 $\pm$ 0.10 ^{ab}

*Values are expressed as mean $\pm$ SD of triplicate determination. Different alphabet superscript within the same column are significantly different ($P=0.05$). Same superscripts within the column indicate no significant difference. STLB- Mixed yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrukii* ssp *bulgaricus*; LA-5- *Lactobacillus acidophilus* LA-5; BB-12- *Bifidobacterium animalis* ssp. *lactis* BB-12

The control yoghurt (without any leaf extract) showed fermentation times of 4.6 h for STLB, 23.3 h for *Lactobacillus acidophilus* LA-5, 47 h for *Bifidobacterium animalis* ssp. *lactis* BB-12, and 4.5 h for the combined culture. Among the green leafy vegetables, curry leaves stood out as the most effective,

significantly reducing the fermentation time for all strains, especially when used in combination. For STLB, *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* ssp. *lactis* BB-12 and combined cultures, the fermentation time decreased to 3.1, 17.3, 28.7 and 2.8 h respectively, which was the shortest

fermentation time compared to all other green leafy vegetables at the same concentration, highlighting its strong potential as a prebiotic enhancer in yoghurt formulations. Spinach and coriander leaves at 50% concentration reduced fermentation time to 18 h for *Lactobacillus acidophilus* LA-5.

Among all the green leafy vegetables tested, only curry leaves had significant impact on reducing the fermentation time for *Bifidobacterium animalis* ssp. *lactis* BB-12, with a marked decrease to 28.7 h at 50% concentration. Others showed no impact on the fermentation time for *Bifidobacterium animalis* ssp. *lactis* BB-12, with times remaining close to the control (47 h). This indicates that these green leafy vegetables did not significantly influence the fermentation process by this specific

strain. Phenolic components and bioactive compounds present in curry leaves might be responsible for the stimulated activity of STLb, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 as studies have shown that polyphenols can mimic prebiotics and encourage the proliferation of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. These prebiotic-like properties of polyphenols can enhance bacterial fermentation efficiency, leading to a reduction in fermentation time^[41].

DMC results showed a significant increase in bacterial populations for all probiotic strains and combinations when supplemented with juice extracts except coriander leaves extracts (Table 2).

Table 2: Direct Microscopic Count of yoghurt in presence of green leafy vegetable extracts

Green leafy vegetables	Concentration (%)	DMC (log ₁₀ cells/mL)			
		STLB (2%)	LA-5 (5%)	BB-12 (5%)	STLB+LA-5+BB-12 (2%+2.5%+2.5%)
Control (No extract)	0	7.32 ± 0.13 ^{abc}	6.88 ± 0.01 ^a	6.70 ± 0.1 ^{abc}	7.36 ± 0.10 ^{def}
Spinach	10	6.97 ± 0.01 ^{ef}	5.61 ± 0.01 ^h	5.46 ± 0.25 ⁱ	7.12 ± 0.01 ^{ef}
	25	7.10 ± 0.10 ^{de}	5.70 ± 0.005 ^h	5.60 ± 0.01 ^{hi}	7.07 ± 0.01 ^{ef}
	50	7.16 ± 0.05 ^{cd}	5.92 ± 0.02 ^g	5.82 ± 0.02 ^h	7.23 ± 0.05 ^{def}
Radish leaves	10	6.66 ± 0.05 ^g	6.11 ± 0.02 ^f	6.20 ± 0.10 ^{fg}	7.45 ± 0.01 ^{def}
	25	6.82 ± 0.02 ^{fg}	6.22 ± 0.02 ^{ef}	6.13 ± 0.11 ^g	7.0 ± 0.51 ^f
	50	7.21 ± 0.02 ^{cd}	6.34 ± 0.02 ^e	6.30 ± 0.01 ^{efg}	7.50 ± 0.1 ^{cdef}
Coriander leaves	10	6.80 ± 0.07 ^{fg}	6.51 ± 0.01 ^d	6.30 ± 0.1 ^{efg}	6.21 ± 0.02 ^g
	25	6.22 ± 0.025 ⁱ	6.71 ± 0.01 ^{bc}	6.50 ± 0.01 ^{bcd}	6.99 ± 0.01 ^f
	50	6.08 ± 0.01 ⁱ	6.91 ± 0.01 ^a	6.73 ± 0.02 ^{ab}	6.22 ± 0.01 ^g
Curry leaves	10	7.42 ± 0.03 ^{ab}	6.53 ± 0.2 ^d	6.51 ± 0.02 ^{bcd}	8.1 ± 0.26 ^{ab}
	25	7.43 ± 0.01 ^{ab}	6.82 ± 0.02 ^{ab}	6.68 ± 0.07 ^{abc}	8.04 ± 0.39 ^{abc}
	50	7.50 ± 0.1 ^a	6.94 ± 0.005 ^a	6.88 ± 0.01 ^a	8.16 ± 0.28 ^a
Mint leaves	10	7.44 ± 0.02 ^{ab}	6.56 ± 0.05 ^{cd}	6.41 ± 0.02 ^{cdefg}	7.31 ± 0.01 ^{def}
	25	7.20 ± 0.1 ^{cd}	6.70 ± 0.005 ^{bc}	6.36 ± 0.2 ^{defg}	7.44 ± 0.02 ^{def}
	50	6.46 ± 0.01 ^h	6.82 ± 0.02 ^{ab}	6.70 ± 0.1 ^{abc}	7.78 ± 0.07 ^{abcd}

*Values are expressed as mean ± SD of triplicate determination. Different alphabet superscript within the same column are significantly different ($P=0.05$). Same superscripts within the column indicate no significant difference. STLb- Mixed yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrückii* ssp. *bulgaricus*; LA-5- *Lactobacillus acidophilus* LA-5; BB-12- *Bifidobacterium animalis* ssp. *lactis* BB-12

Curry leaves, in particular, enhanced the growth of both yoghurt bacteria and probiotic bacteria, especially in the combined form (STLB+LA-5+BB-12), which reached the highest value among all tested leaves. Across the different concentrations of spinach extracts, the DMC counts decreases compared to the control, especially for *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12, which show significantly lower values at 10% concentration (5.61 and 5.46 log₁₀ cells/mL, respectively). Higher concentrations of spinach (25% and 50%) slightly improve the counts but remain lower than the control. In line with our findings, Fadaei *et al.*^[42] investigated the cell viability of yoghurt starter cultures, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus*, under refrigeration in yoghurts supplemented with Spirulina powder (at 0.3, 0.5, and 0.8% w/w) and spinach (at 10 and 13% w/w). The study revealed that the addition of spinach at those concentrations did not significantly enhance the viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Streptococcus thermophilus* in the yoghurt samples. Furthermore, no significant differences were observed in the viability of the yoghurt starter cultures or probiotics between the two spinach concentrations.

Radish leaves, especially at higher concentrations (50%), show a positive effect on STLb and the combination of strains (7.2 and 7.5 log₁₀ cells/mL, respectively). Coriander leaves extract show a negative effect on bacterial count, especially at higher concentrations (50%). STLb (6.08 log₁₀ cells/mL) and

combination of strains (6.22 log₁₀ cells/mL) show a significant reduction, which is lower than the control. Shariati *et al.*^[43] investigated the impact of coriander leaves extract (CE) on the viability of *Lactiplantibacillus plantarum* LS5 in drinking yoghurt. CE was added at concentrations of 0, 0.05, 0.1, and 0.25%. The study found that adding CE at 0.05% enhanced probiotic viability, likely due to the presence of prebiotic compounds, including phenolics. Consequently, a greater reduction in pH and an increase in acidity were observed in samples with 0.05% CE due to enhanced bacterial growth and lactic acid production. However, at higher concentrations (0.1% and 0.25%), the acidity slightly decreased compared to 0.05%, possibly due to the antimicrobial properties of CE attributed to certain phenolic and antibacterial compounds. This is consistent with our findings, where using 1% coriander leaves extract at concentrations of 10%, 25%, and 50% potentially led to inhibition of yoghurt culture and probiotic culture used.

Curry leaves extract has the strongest significant positive impact on all the bacterial strains used, especially at the highest concentration (50%), where STLb (7.5 log₁₀ cells/mL) and the combination of strains (8.16 log₁₀ cells/mL) show the highest counts among all green leafy vegetables. *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 also perform well with curry leaves, reaching highest values (6.94 and 6.88 log₁₀ cells/mL respectively), indicating the prebiotic potential of curry leaves, particularly at higher concentrations. Mint leaves extract showed a varied effect on different strains. While STLb count reduced with increasing

concentrations, the growth of other two probiotic strains, are supported, indicating that mint may have prebiotic potential but inhibit the growth of STLB used for yoghurt production. However the combination improved to 7.78 log₁₀ cells/mL compared to control.

The different green leafy vegetables used in the study have varying effects on the growth of the yoghurt culture (STLB),

Lactobacillus acidophilus LA-5, *Bifidobacterium animalis* ssp. *lactis* BB-12 and their combinations and are primarily reflected in the acidity, as the production of lactic acid during fermentation is a direct result of bacterial growth and activity. The results of titratable acidity evaluation are presented in Table 3.

Table 3: Titratable acidity of yoghurt in presence of green leafy vegetable extracts

Green leafy vegetables	Concentration (%)	Acidity (%LA)			
		STLB (2%)	LA-5 (5%)	BB-12 (5%)	STLB+LA-5+BB-12 (2%+2.5%+2.5%)
Control (No extract)	0	0.64± 0.01 ^a	0.47 ± 0.02 ^{bcd}	0.46 ± 0.01 ^b	0.59 ± 0.05 ^{abcde}
Spinach	10	0.47± 0.01 ^{def}	0.42 ± 0.02 ^{bcd}	0.43 ± 0.01 ^b	0.46 ± 0.01 ^{hij}
	25	0.46± 0.01 ^{ef}	0.43 ± 0.01 ^{bcd}	0.44 ± 0.01 ^b	0.47 ± 0.02 ^{ghij}
	50	0.51± 0.01 ^{cde}	0.43 ± 0.02 ^{bcd}	0.46 ± 0.01 ^b	0.49 ± 0.04 ^{fghij}
Radish leaves	10	0.51± 0.005 ^{cde}	0.41 ± 0.005 ^{cd}	0.42 ± 0.02 ^b	0.52 ± 0.005 ^{defghij}
	25	0.52± 0.01 ^{cde}	0.44 ± 0.01 ^{bcd}	0.44 ± 0.01 ^b	0.53 ± 0.15 ^{cdefghi}
	50	0.47± 0.06 ^{def}	0.46 ± 0.01 ^{bcd}	0.42 ± 0.02 ^b	0.54 ± 0.005 ^{cdefgh}
Coriander leaves	10	0.52± 0.01 ^{cde}	0.41 ± 0.005 ^d	0.46 ± 0.01 ^b	0.52 ± 0.01 ^{defghij}
	25	0.52± 0.01 ^{cde}	0.43 ± 0.01 ^{bcd}	0.42 ± 0.01 ^b	0.51 ± 0.01 ^{efghij}
	50	0.43± 0.005 ^f	0.48 ± 0.05 ^{bcd}	0.44 ± 0.02 ^b	0.45 ± 0.01 ^{ij}
Curry leaves	10	0.54± 0.01 ^{cd}	0.46 ± 0.01 ^{bcd}	0.44 ± 0.005 ^b	0.61 ± 0.02 ^{abcd}
	25	0.61± 0.005 ^{ab}	0.49 ± 0.01 ^{bc}	0.45 ± 0.02 ^b	0.62 ± 0.03 ^{abc}
	50	0.67± 0.005 ^a	0.59 ± 0.01 ^a	0.53 ± 0.02 ^a	0.66 ± 0.02 ^a
Mint leaves	10	0.64± 0.05 ^a	0.43 ± 0.02 ^{bcd}	0.45 ± 0.005 ^b	0.44 ± 0.02 ^{ij}
	25	0.67± 0.02 ^a	0.46 ± 0.01 ^{bcd}	0.45 ± 0.02 ^b	0.57 ± 0.03 ^{abcdef}
	50	0.61 ± 0.01 ^{ab}	0.49 ± 0.05 ^b	0.46 ± 0.01 ^b	0.56 ± 0.01 ^{bcddefg}

*Values are expressed as mean ± SD of triplicate determination. Different alphabet superscript within the same column are significantly different ($P=0.05$). Same superscripts within the column indicate no significant difference. STLB- Mixed yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrückii* ssp. *bulgaricus*; LA-5- *Lactobacillus acidophilus* LA-5; BB-12- *Bifidobacterium animalis* ssp. *lactis* BB-12; LA- Lactic acid.

Spinach lowers the acidity to 0.51% LA at 50% concentration compared to the control sample having 0.64% LA acidity, suggesting spinach does not significantly enhance STLB activity. In case of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12, acidity ranged between 0.42 to 0.46% LA, which is similar to the control, indicating spinach has little effect on their growth and acid production. At 50% concentration of spinach leaves extract, the combination of all the three bacteria showed acidity 0.49% LA which is lower than the control sample (0.59% LA), suggesting spinach does not increase the synergistic activity of probiotic strains used. In the presence of radish leaves extract, the acidity remains within a lower range (0.41% - 0.46%) when fermented with *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 individually, similar to the control, indicating that radish leaves have minimal impact on these strains.

Acidity of the sample inoculated with STLB at 50% concentration of coriander leaves extract was found to be 0.43% LA, much lower than the control (0.64%), indicating coriander leaves do not enhance STLB growth as much as the control. For *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12, acidity (0.41% - 0.46%) was similar to the control, showing little impact of coriander leaves extract on these strains. Acidity reaches 0.52% with mixed culture, which is lower than the control (0.59%), indicating coriander leaves have a weaker effect on all the strains used. Curry leaves extract resulted in significantly higher acidity of 0.67, 0.59 and 0.53% LA respectively at 50% compared to the control, indicating strong enhancement in growth and fermentation by STLB, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 individually. In combination, acidity reaches 0.66%, surpassing the control (0.59%), showing strong synergistic effects when curry leaves extract are combined with probiotics and met FSSAI standards, which mandate a minimum

titratable acidity of 0.6% lactic acid for yoghurt. The increase in acidity is due to bacterial activity converting lactose into organic acids, mainly lactic acid, indicating higher activity in the presence of the phenolic rich leaf extract [10].

The impact of mint leaves extract on STLB, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 was similar to the control. A reduction in titratable acidity was noted when combined culture was used, suggesting that the increased acidity observed when STLB culture, *Lactobacillus acidophilus* LA-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12 were inoculated individually could be due to the inherent acidity of the mint extracts. This acidity may have inhibited the growth of the bacteria when they were used in combination. In a study reported by Al-Shawi [44] explored the impact of inulin and mint extracts in pH and titratable acidity of low-fat synbiotic yogurt fermented with *Lactobacillus acidophilus* and found that addition of inulin led to a reduction in pH values and an increase in titratable acidity and the highest titratable values were observed in inulin-treated samples with alcoholic (1.23% LA) and aqueous mint extract (1.17% LA). These values were higher than those in our study and may be attributed to the presence of inulin, an effective prebiotic. Additionally, our results align with Hussain [45], who found that adding aqueous mint extract at varying levels (1, 2.5, and 4% v/v) did not significantly influence the pH or acidity of yoghurt, suggesting that mint extract does not affect the activity of starter bacteria responsible for lactic acid production.

The differences in acidity can be attributed to the varying levels of bioactive compounds, such as phenolic compounds and fiber, present in the green leafy vegetables, which impact the fermentation process.

Assessment of viability by spread plate method

The results of viability assessment are given in Table 4.

Table 4: Viability of yoghurt and probiotic cultures in presence of green leafy vegetables

Green leafy vegetables	Viability count ($\log_{10}$ CFU/mL)		
	STLB	LA-5	BB-12
Control (No GLVP)	7.5 ± 0.01^a	8.3 ± 0.10^a	7.9 ± 0.10^b
Spinach	5.6 ± 0.10^c	7.6 ± 0.10^a	Nil
Radish leaves	7.4 ± 0.10^{ab}	8.4 ± 0.10^a	6.6 ± 0.10^c
Coriander leaves	7.3 ± 0.10^{ab}	7.9 ± 0.10^a	5.9 ± 0.10^d
Curry leaves	7.2 ± 0.10^b	8.5 ± 0.10^a	8.3 ± 0.10^a
Mint leaves	Nil	5.0 ± 1.0^b	Nil

*Values are expressed as mean $\pm$ SD of triplicate determination. Different alphabet superscript within the same column are significantly different ($P=0.05$). Same superscripts within the column indicate no significant difference. STLB- Mixed yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbruckii* ssp *bulgaricus*; LA-5- *Lactobacillus acidophilus* LA-5; BB-12- *Bifidobacterium animalis* ssp. *lactis* BB-12;

The viability of STLB, *Lactobacillus acidophilus* LA-5, and *Bifidobacterium animalis* ssp. *lactis* BB-12 in control group without any GLVP was found to be 7.5, 8.3, and 7.9 $\log_{10}$ CFU/mL respectively and this served as a reference for comparing the effects of the green leafy vegetables. For STLB, radish leaves, coriander leaves, and curry leaves showed comparable results to the control. Radish leaves had a viability of 7.4 $\log_{10}$ CFU/mL, while coriander leaves had 7.3 $\log_{10}$ CFU/mL, and curry leaves showed 7.2 $\log_{10}$ CFU/mL. These values indicate that all three green leafy vegetables support the growth of STLB, with slight variations in their effectiveness. In contrast, spinach had a significantly lower STLB viability of 5.6 $\log_{10}$ CFU/mL, indicating it provides much less support for STLB. Mint leaves at 5% level did not support any STLB growth, as indicated by the absence of viable colonies.

For *Lactobacillus acidophilus* LA-5, curry leaves were the most supportive, yielding a viability of 8.5 $\log_{10}$ CFU/mL, which was higher than the control. This is in accordance with the study reported by Bhatt *et al.* (2020) that signifies the prebiotic potential of curry leaves in supporting probiotic growth. Their study found that freeze-dried hydro-ethanolic extract of curry leaves (1% w/v) enhanced the growth of *Lactobacillus acidophilus* ATCC 4356, comparable to inulin (8.2 $\log_{10}$ CFU/mL). But unlike our study, they observed no significant increase in viability compared to the control. This discrepancy may be due to the strain-specific nature of prebiotic activity, as different probiotic strains can respond variably to the

same prebiotic compound based on their unique metabolic characteristics and substrate preferences [46, 47].

Radish leaves also showed strong support for *Lactobacillus acidophilus* LA-5, with viabilities of 8.4 $\log_{10}$ CFU/mL. Coriander leaves showed slightly lower support at 7.9 $\log_{10}$ CFU/mL, but this was still within an acceptable range. Spinach supported *Lactobacillus acidophilus* LA-5 to a lesser extent, with a viability of 7.6 $\log_{10}$ CFU/mL, while mint leaves were the least supportive, with only 5.0 $\log_{10}$ CFU/mL.

For *Bifidobacterium animalis* ssp. *lactis* BB-12, curry leaves again demonstrated the strongest support, with a viability of 8.3 $\log_{10}$ CFU/mL, surpassing the control. Radish leaves showed moderate support, with a viability of 6.6 $\log_{10}$ CFU/mL, while coriander leaves (5.9 $\log_{10}$ CFU/mL) exhibited weak support. Spinach and mint leaves did not support *Bifidobacterium animalis* ssp. *lactis* BB-12 growth, as indicated by the absence of viable colonies. This might be due to the ability of polyphenols particularly phenolic acids and flavonoids, at higher concentrations to bind to the thiol groups at the active site of various microbial enzymes makes them inactive, thus inhibiting the growth of microbes [48].

Prebiotic Activity Score (PAS) and Prebiotic Index (PI) of green leafy vegetables

Fig 1 and Fig 2 present the PAS and PI for different green leafy vegetables.

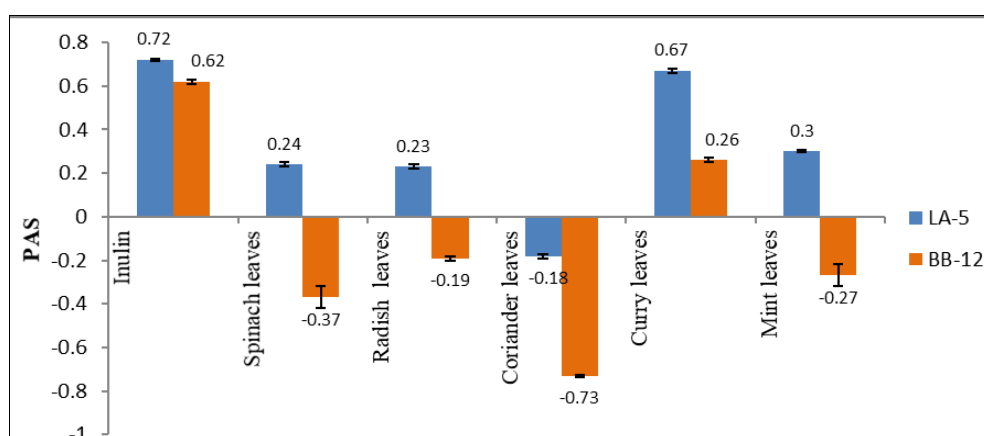


Fig 1: Prebiotic Activity Score (PAS) of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 grown on different green leafy vegetables and inulin (standard prebiotic) at 1% concentration

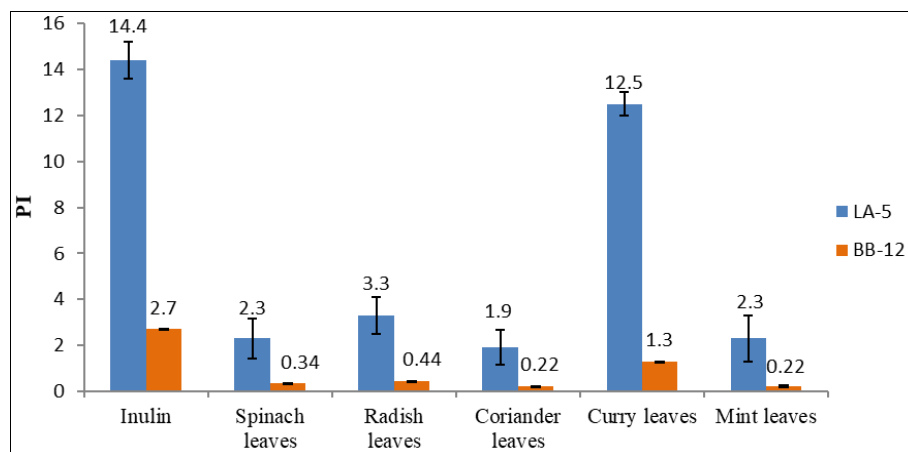


Fig 2: Prebiotic Index (PI) of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 grown on different green leafy vegetables and inulin (standard prebiotic) at 1% concentration

Higher PAS indicate higher relative growth of the probiotic and/or lower relative growth of the enteric pathogenic bacteria. The highest PAS for *Lactobacillus acidophilus* LA-5 was obtained when grown on commercial inulin (0.72) followed by curry leaves (0.67), which is nearly identical to inulin's PAS ($P=0.05$) and the lowest score was observed when grown with coriander leaves extract (-0.18). For *Bifidobacterium animalis* ssp. *lactis* BB-12, although none of the green leafy vegetables reached the performance levels of inulin with PAS 0.62, curry leaves still emerged as the best option among the studied green leafy vegetables with a PAS of 0.26. All the other green leafy vegetables scored negative PAS values for *Bifidobacterium animalis* ssp. *lactis* BB-12. A negative PAS means that the test strain showed poorer growth on the prebiotic compared to its growth on glucose, or that its growth on the prebiotic was lower than that of harmful enteric bacteria on the same substrate [36]. In a previous study, PAS values for *L. acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 were found to be 1.2 and 1.69, which is higher than our findings, when grown in MRS supplemented with 10 mg/mL (1%) of inulin using *Escherichia coli* ATCC 25922 as enteric species, whereas the PAS obtained for *Lactobacillus reuteri* ATCC 23272 and *Bifidobacterium breve* 286 grown on inulin was found to be 0.18 and 0.28 respectively when paired with enteric bacteria *E. coli* ATCC 25922 [49]. In another research carried out by Huebner *et al.*, PAS values for probiotic *L. acidophilus* NCFM, *L. acidophilus* 33200 and *Bifidobacterium bifidum* NCI calculated by using enteric mixture of *E. coli* strains; ECOR 1, ECOR 2, and ECOR 22 in a 1:1:1 ratio, grown on commercial inulin were found to be 0.12, -0.2 and -1.17 respectively, which was lower than the values obtained in the present study [36].

The variation in PAS can be explained by the fact that strains from the same species often show significant differences in their PAS for the same prebiotic, including inulin. This indicates that even closely related strains can have distinct metabolic capabilities. The ability of probiotics to utilize prebiotics depends on the presence of specific genes encoding enzymes and transport systems required for breaking down and absorbing the prebiotic, which may be present in some strains but not in others, resulting in varying levels of prebiotic activity [36]. Additionally, the structure of prebiotic compounds affects both their solubility and the capacity of bacteria to metabolize them, which also varies between strains [48].

The prebiotic index provides a quantitative measure for comparing different prebiotic substrates. For both *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-

12, the highest PI value was obtained when grown on inulin (14.4 and 2.7 respectively). Comparing the prebiotic potential of various green leafy vegetables with the standard prebiotic inulin, curry leaves stand out as the closest match, with PI values 12.5 and 1.3 for *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 respectively. The other green leafy vegetables, such as spinach, radish leaves, coriander leaves, and mint leaves, had lower PI values, indicating lesser but still measurable prebiotic activity, particularly for *Bifidobacterium animalis* ssp. *lactis* BB-12 where values remain below 1. According to a study carried out by Dawood *et al.* identified the PI values for probiotic *L. casei* MSD102 and *L. rhamnosus* ML57 grown on 2% inulin as 1.1 and 0.7 respectively [38]. A PI value greater than 1 indicates that the tested prebiotic promotes the growth of the microorganism more effectively than the control carbohydrate [49]. Low PI values occur when a probiotic strain exhibits poorer growth in the presence of a prebiotic (extracts or purified compounds) compared to its growth on glucose [38].

Modified prebiotic index (PI_m) is a measure of how well a substance promotes the growth of beneficial gut bacteria and inhibit the growth of harmful enteric bacteria. A higher PI_m value indicates a better prebiotic potential. Fig 3 presents the PI_m for different green leafy vegetables. As expected, inulin has the highest PI_m value (0.38), given that it's a well-established prebiotic. Among the green leafy vegetables, curry leaves exhibit the highest modified prebiotic index (0.3), suggesting a strong potential to support beneficial bacteria in the gut. Radish leaves show slightly higher PI_m (0.18) than other green leafy vegetables, though it's much lower than inulin. Both spinach and mint leaves shared the same PI_m value (0.17). Coriander leaves has the lowest PI_m value (0.09), indicating less prebiotic potential compared to the other green leafy vegetables. Palframan *et al.* reported PI_m value of 0.38 and -1.2 for populations of bifidobacteria, lactobacilli, bacteroides and clostridia (histolyticum subgroup), when grown on inulin at 1% and 2% (w/v) level respectively at pH 6.8, indicating the effect of concentration of prebiotics in stimulating desirable bacterial growth [50]. A study conducted by Cardarelli *et al.* reported the development of a cheese with the potential to generate a synbiotic effect by incorporating probiotic *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BL-04, and a prebiotic blend (0.25% w/v) consisting of equal parts inulin, fructooligosaccharides, and honey and the PI_m value was calculated as 1.82 using desirable bacterial groups bifidobacteria, lactobacilli, and eubacteria and undesirable bacterial groups

Bacteroides spp., *Clostridium histolyticum*, *Escherichia coli*, and *Desulfovibrio* spp., indicating a favorable environment for the

growth of beneficial bacteria over harmful ones [39].

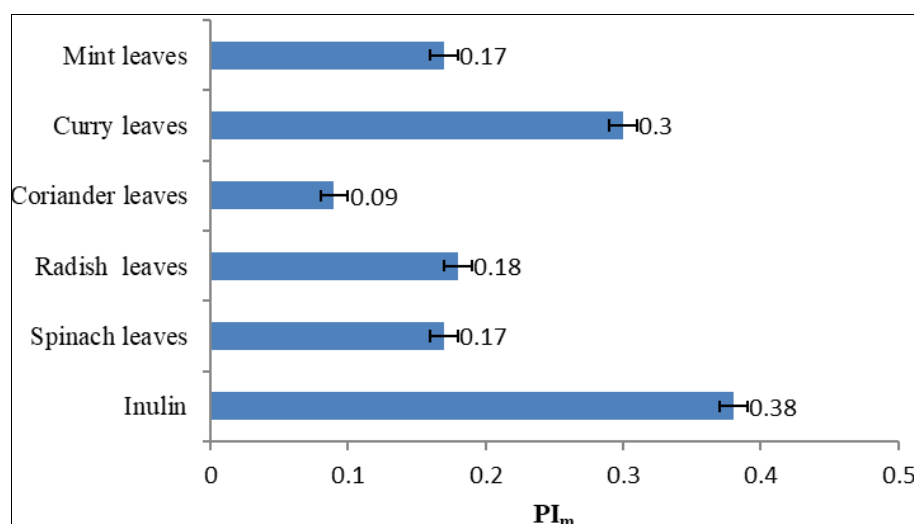


Fig 3: Modified prebiotic index (PI_m) of different green leafy vegetables and inulin (standard prebiotic) at 1% concentration.

Fig 4 provides a comprehensive comparison of the prebiotic potential of six green leafy vegetables based on key parameters: fermentation time, DMC ($\log_{10}$ cells/mL), acidity (%LA),

viability counts ($\log_{10}$ CFU/mL), PAS, PI and PI_m for the respective probiotics.

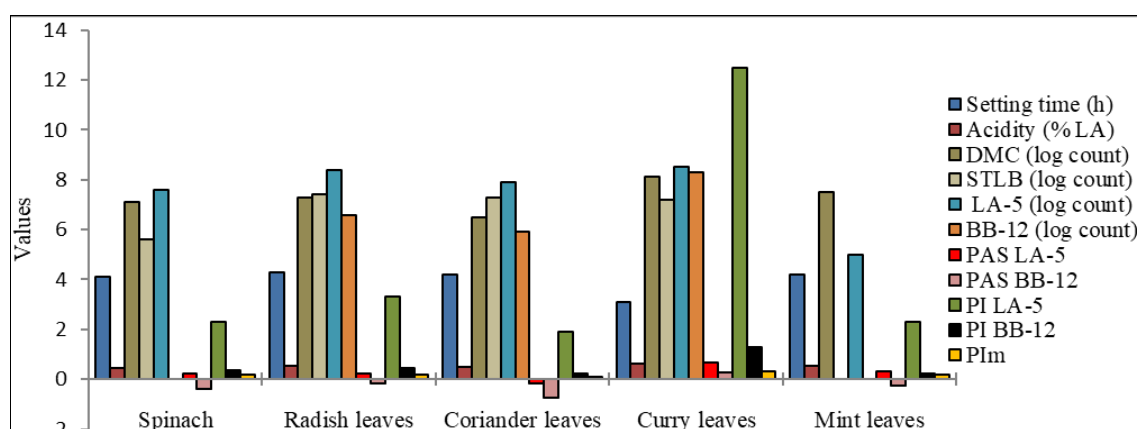


Fig 4: Comparative prebiotic potential of the green leafy vegetables

Curry leaves show the highest prebiotic potential, with the lowest fermentation time (2.8 h), the highest PAS (0.67), PI (12.5), and strong viability counts across strains. Radish leaves also exhibit strong prebiotic potential, with moderate fermentation times, decent viability, and higher PI scores compared to other green leafy vegetables. Spinach and mint leaves perform moderately, with similar PAS, PI and PI_m values with acceptable fermentation times and DMC. They exhibited lower viability for STL B and *Lactobacillus acidophilus* LA-5 and inhibited the growth of *Bifidobacterium animalis* ssp. *lactis* BB-12 at 5% level. Coriander leaves has the lowest prebiotic potential, with the lowest PAS and PI_m, though its viability counts are moderate. This indicates that coriander leaves do not have the potential to inhibit the growth of enteric *E. coli*. Thus curry leaves rank highest in prebiotic potential for both *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 strains, followed by radish leaves, making them the best candidates for synbiotic yoghurt formulation.

Conclusion

The study highlights curry leaves as the most effective among the tested Indian indigenous green leafy vegetables for

enhancing the growth and activity of probiotics in synbiotic yoghurt formulations. Their ability to reduce fermentation time and selectively promote the growth of beneficial probiotic strains such as *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12, while also inhibiting harmful bacteria like *E. coli*, makes them a promising alternative to conventional prebiotics like inulin. Radish leaves similarly supported probiotic growth and reduced fermentation time, making both leaves strong candidates for functional food applications. While these green leafy vegetables offer significant prebiotic potential and are rich in essential nutrients, careful consideration of their oxalates, alkaloids, saponins or phytates content which, in high amounts, could interfere with nutrient absorption or contribute to kidney stones and allergenic potential is necessary to ensure safe consumption. Hence, ensuring safe consumption and determining appropriate dosages is critical when incorporating these green leafy vegetables into synbiotic formulations. Further research is needed to optimize their concentrations and assess their synergistic effects in synbiotic dairy products, paving the way for new, health-promoting food innovations.

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