Development and shelf life evaluation of plant milk based yogurt enriched with *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei*

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Abstract
Consumer interest in plant-based dairy substitutes has increased, which leads to the development of new non-dairy-based products like oat milk yogurt. Oat milk is rich in essential nutrients, dietary fibers and β-glucans. It is an ideal substrate for probiotic fermentation, offering a creamy texture and enhanced nutritional value compared to other plant-based milk alternatives. The current study explores the compatibility of Lacticaseibacillus rhamnosus and Lacticaseibacillus paracasei in the consortium and their use as starter cultures in oat milk-based yogurt production. This study further evaluated the various physiological and biological changes of the developed yogurt during storage at 4°C throughout 10 days.

The 5% (v/v) probiotic consortium of L. rhamnosus and L. paracasei (1:1) produced thick and creamy yogurt which when stored under refrigeration at 4°C showed a significant increase in lactobacillus count and sensory attributes of the yogurt, peak at day 4, providing the best aroma, taste and texture to the oat milk yogurt. Overall, the findings support the development of a non-dairy, vegan oat milk yogurt enriched with probiotics, offering potential health benefits and enhanced consumer acceptability.

Keywords: Oat milk, Probiotics, Non-dairy based products, Vegan.

Introduction
Consumer interest is growing more in plant-based dairy substitutes which sparked the creation of novel non-dairy products like oat milk yogurt, almond milk yogurt and such others, providing a palatable option for those who are lactose intolerant, allergic to dairy, or vegan. Oat milk is rich in vital nutrients, dietary fibers and β-glucans and excellent substrate for probiotic fermentation[12]. Oat milk has a creamy texture and more nutritional value as compared to other plant-based milk options, so it is a viable substrate for yogurt manufacturing[2].

The World Health Organization defines probiotics as live microorganisms that provide the host with health benefits when given in adequate amounts[4] and are increasingly included in fermented foods because of their health-promoting activities. *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei* are well-studied probiotic strains that positively affect gut health and immune modulation and prevent gastrointestinal disorders[11]. These strains can ferment any plant-based substrates such as oat milk and are also able to enhance the nutritional profile of the resulting yogurt. Even though oat milk yogurt provides a good alternative to conventional dairy products, the stability and shelf life of plant-based probiotic-enriched products remain important determinants of their acceptability by consumers and economic viability.

For maintaining product quality, it is important to closely monitor the metabolic activity of probiotics in the products during storage as well as any potential modifications to the texture, flavor and microbiological safety[6]. Developing a bacterial consortium with *L. rhamnosus* and *L. paracasei*, provides a synergistic method for increasing the probiotic levels in plant-based yogurts. Co-culturing these strains could increase their potential to survive during fermentation and storage and also to improve the sensory and nutritional values of the final end product.

However, it is still difficult to maintain probiotic viability during storage in non-dairy substrates like oat milk because of factors such as pH changes, oxygen exposure and temperature fluctuations[9].

The purpose of this study is to assess the stability of the yogurt during storage and to investigate the formation of a bacterial consortium with *L. rhamnosus* and *L. paracasei* for the production of oat milk yogurt. The focus of the research is to determine the yogurt's microbial viability, physico-chemical characteristics and sensory qualities during refrigeration. The findings of this study may provide significant new insights into the manufacturing of stable, plant-based yogurts enriched with probiotic and the variables affecting their shelf life.

Material and Methods
Preparation of oat milk: Oat milk was prepared using commercially available oat flakes and water. Oat flakes were blended with water at a ratio of 1:3 (w/v) and then filtered through a muslin cloth to obtain oat milk. The oat milk was pasteurized at 85°C for 15 minutes to eliminate any potential microbial contaminants[3].

Table 1
Probiotic isolates and their gene accession number used in this study

S.N.	Isolate code	BLAST search result	NCBI gene accession number
1	NHC4	*Lacticaseibacillus rhamnosus*	PP893081
2	NSD4	*Lacticaseibacillus paracasei*	PP911495

Probiotic cultures: *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei* were previously isolated from the various ethnic fermented foods of Gujarat, India (Table 1). *L. rhamnosus and L. paracasei* were individually grown in MRS media at 37°C for 24 hours and then centrifuged at 4,000 rpm for 10 minutes. Collect the cell pellets, rinse twice with sterile phosphate-buffered saline (PBS) and then resuspend in sterile PBS to obtain the desired concentration (10^8 CFU/mL)[5].

Consortium preparation: The bacterial compatibility study required for the preparation of consortia involved two tests: coexistence and antagonistic activity. In the coexistence test, isolates were cross-streaked on MRS agar to observe any antagonism after incubation[7]. For the antagonistic activity test, supernatants from each isolate's culture were tested against other strains by inoculating cells on MRS agar and applying supernatants in wells[8]. *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus paracasei* were shown compatible by these two tests and grown individually in MRS broth at 37°C for 24 hours and then were centrifuged at 4,000 rpm for 10 minutes. The resulting cell pellets were resuspended in sterile phosphate-buffered saline. Equal volumes of *L. rhamnosus* and *L. paracasei* were mixed to form the probiotic consortium[6].

Yogurt preparation: 1:1 mixture of *L. rhamnosus* and *L. paracasei* was added to the oat milk at a final concentration of 1% to 5% (v/v). The inoculated oat milk was then incubated at 37°C for 24 hours. After fermentation, the yogurt was cooled to 4°C and stored for further analysis.

Storage study: The prepared oat milk yogurt was stored at 4°C and sampled at 1, 4, 7 and 10 days. During each sampling interval, the following parameters were evaluated:

Microbial analysis: The viability of *L. rhamnosus* and *L. paracasei* and other contaminants were determined using plate count methods. Samples were diluted and plated on MRS agar, potato dextrose agar and nutrient agar. Colonies were counted after incubation at 37°C for 48 hours[1].

pH measurement: The pH of the oat milk yogurt was measured at each time point using a calibrated digital pH meter under cooling conditions (4°C).

Organoleptic evaluation: Sensory attributes such as flavour, texture and overall acceptability were assessed using a panel of 10 evaluators. The evaluation was done using a 9-point hedonic scale[10].

Statistical Analysis: The data are the means of three separate analyses and the results are reported as means±standard deviations (M±SD). Data were analyzed using Minitab Statistical Program ver. 17. Analysis of Variance (ANOVA) was used to assess the significance of differences across time points, followed by Tukey's post-hoc test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

Oat milk preparation: The oat was successfully prepared using a 1:3 (w/v) ratio of commercially available oat flakes and R/O water. Pasteurized oat milk exhibited a creamy consistency suitable for probiotic fermentation and preparation of non-dairy yogurt (Fig. 1).

Consortium preparation: *L. rhamnosus* and *L. paracasei* were shown to be compatible by the antagonistic activity and co-existence test; no discernible antagonistic behavior was seen during cross-stamping on MRS agar (Fig. 2C). Additionally, the supernatants of both isolates showed no inhibitory effect on each other when applied to MRS agar, confirming the absence of antagonistic activity (Fig. 2A and B). These results confirmed that a stable probiotic consortium could be formed by mixing equal volumes of *L. rhamnosus* and *L. paracasei.*

Yogurt Preparation: The oat milk was inoculated with 1:1 mixture of *L. rhamnosus* and *L. paracasei* at various concentrations ranging from 1% to 5% (v/v). Fermentation at 37°C for 24 hours with inoculum size 5% produced a thickened yogurt with characteristic aroma, taste, color, mouth feel and texture (Table 2, fig. 3).

Storage study

Microbial analysis: The microbial analysis was performed to investigate the survival of starter culture species and the existence in the finished product of contaminants and spoilage microbes. The Lactobacillus counts significantly increased during the storage period from 5.14 ± 0.11 on day 1 to 8.93 ± 0.62 log CFU/mL on day 7 (table 3). The viable cell counts 8.97 ± 0.90 log CFU/mL on day 10 were nearly equal to day 7, which may be due to nutrient depletion for Lactobacillus cultures during storage. During the 10 days of storage time, no yeast, mold and coliform counts were detected, which indicates that the oat yogurt was free of fecal contamination (Table 3).

pH Measurement: The pH of the oat milk yogurt decreased slightly from 5.50 ± 0.50 to 4.17 ± 0.15 during the 10 day storage period but is not considered as significant by tukey's test after ANOVA (Table 4). This gradual decrease is typical of probiotic yogurt fermentation where organic acid production continues even during storage.

Sensory Evaluation: The sensory evaluation of oat milk yogurt revealed significant differences in aroma, taste, color, texture and overall acceptability during 10-day refrigerated storage studies at 4℃. The highest rating for all sensory parameters was observed on day 4, after which there was a gradual decline (fig. 4, table 5). The aroma scored 8.00 on day 4 but dropped to 5.9 on day 7. Similarly taste, color, texture, mouthfeel and overall acceptability showed a peak on day 4, followed by a decrease by day 10.

Fig. 1: Preparation of oat milk. (A) 250g oats in the jar; (B) Oats mixed with R/O water in a 1:3 ratio in a clean container; (C) Blending of oats in a grinder; (D) Strain through muslin cloth to get the clarified oat milk; (E) Oats remnants remain after straining of oat milk; (F) Oat milk.

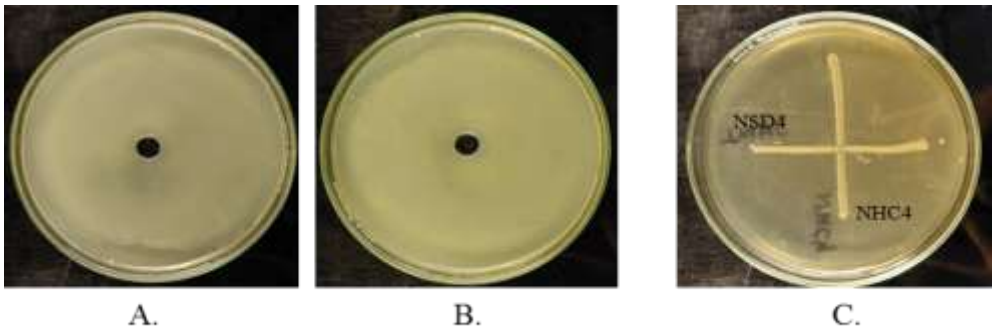

Fig. 2: Bacterial compatibility tests for consortium preparation. (A) Antagonistic activity test with supernatant of *L. rhamnosus*; (B) Antagonistic activity test with supernatant of *L. paracasei*; (C) Coexistence test of *L. rhamnosus* NHC4 and *L. paracasei* NSD4.

Table 2
Sensory scores of different inoculum size batches used for the selection of optimal yogurt formulation

	Consortium concentration				
Sensory parameters	**1%**	**2%**	**3%**	**4%**	**5%**
Aroma	2.66 ± 0.38^{C}	2.83 ± 0.38^{C}	4.33 ± 0.28^{B}	7.00 ± 0.25^{A}	7.75 ± 0.50^{A}
Taste	2.58 ± 0.14^{d}	3.25 ± 0.50^{d}	4.58 ± 0.38^{c}	7.00 ± 0.25^{b}	8.08 ± 0.57^{a}
Color	2.33 ± 0.52^{r}	2.83 ± 0.38^{r}	4.33 ± 0.28^{q}	7.00 ± 0.25^{p}	7.83 ± 0.52^{p}
Mouth-feel	2.58 ± 0.15^{m}	2.83 ± 0.39^{m}	4.33 ± 0.28^{l}	7.00 ± 0.25^{k}	7.67 ± 0.38^{k}
Texture	2.58 ± 0.15^{z}	2.83 ± 0.39^{z}	4.33 ± 0.29^{y}	7.00 ± 0.25^{x}	8.08 ± 0.63^{w}
Overall acceptability	2.17 ± 0.52^{n}	2.58 ± 0.52^{n}	6.67 ± 0.87^{m}	7.67 ± 0.87^{m}	8.34 ± 0.14^{m}

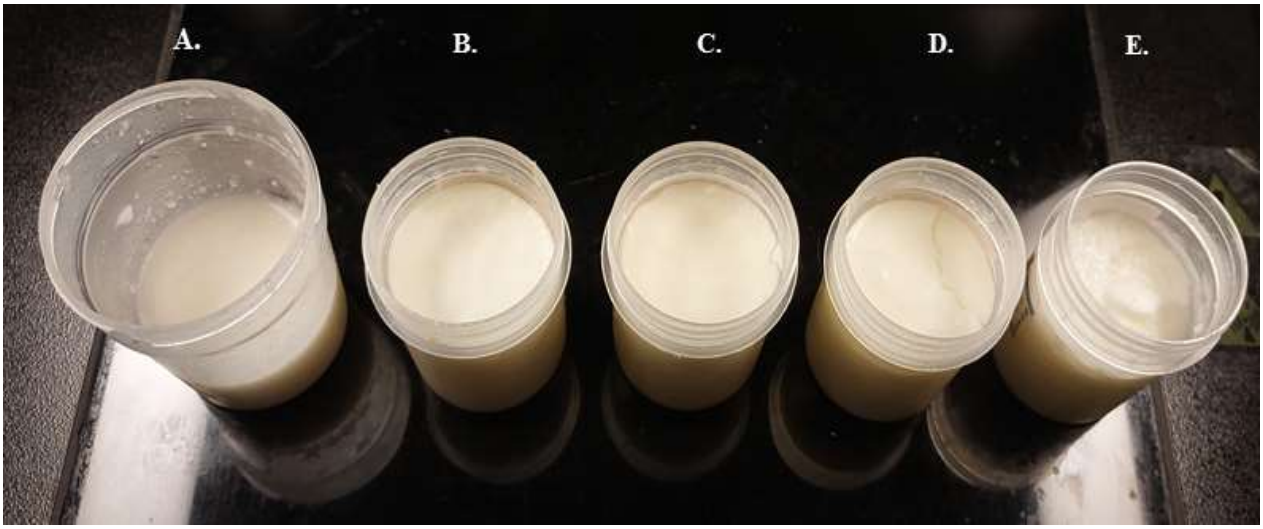

Fig. 3: Yogurt Preparation with different inoculum size 1% to 5% (A to E)

Table 3
Microbial analysis of oat milk yogurt during 10 days' storage study (Refrigeration temp. 4°C)

Storage days	Lactic counts (log CFU/ml)	Yeast and Mold count (log CFU/ml)	Coliforms count (log CFU/ml)
Day 1	5.14 ± 0.11^{B}	Absent	Absent
Day 4	7.99 ± 0.71^{A}	Absent	Absent
Day 7	8.93 ± 0.62^{A}	Absent	Absent
Day 10	8.97 ± 0.90^{A}	Absent	Absent

Table 4
pH analysis of optimized oat milk yogurt during storage for 10 days (Refrigeration temp. 4°C)

Storage days	pH
Day 1	5.50 ± 0.50^{a}
Day 4	4.84 ± 0.76^{a}
Day 7	4.54 ± 0.51^{a}
Day 10	4.17 ± 0.15^{a}

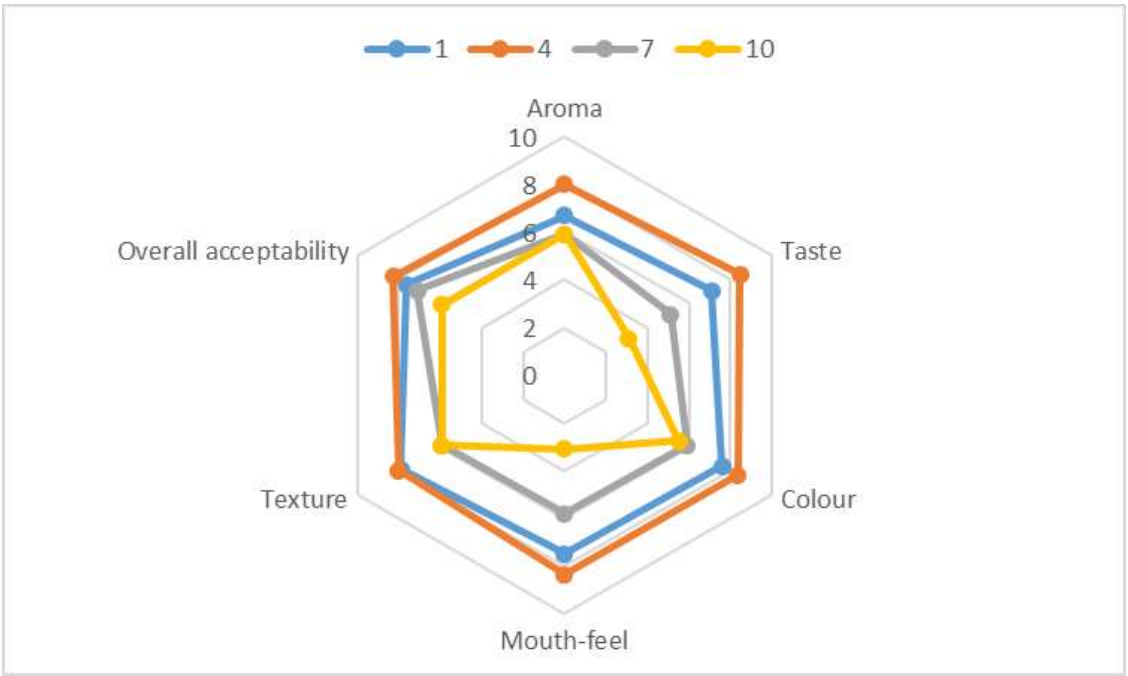

Fig. 4: Sensory evaluation of optimized oat milk yogurt during storage for 10 days

Table 5
Sensory scores of optimized oat milk yogurt during storage for 10 days (Refrigeration temp. 4°C)

Parameter	Storage days	Sensory score
Aroma	Day 1	6.70 ± 1.16^{b}
	Day 4	8.00 ± 1.00^{a}
	Day 7	5.90 ± 0.73^{b}
	Day 10	5.90 ± 0.87^{b}
Taste	Day 1	7.10 ± 0.87^{f}
	Day 4	8.45 ± 0.52^{e}
	Day 7	5.10 ± 0.99^{g}
	Day 10	3.10 ± 1.37^{h}
Colour	Day 1	7.60 ± 0.84^{i}
	Day 4	8.36 ± 0.67^{i}
	Day 7	5.90 ± 0.74^{j}
	Day 10	5.50 ± 0.85^{j}
Mouth-feel	Day 1	7.50 ± 1.08^{k}
	Day 4	8.36 ± 0.80^{k}
	Day 7	5.80 ± 0.78^{l}
	Day 10	3.10 ± 1.37^{m}
Texture	Day 1	7.90 ± 0.74^{n}
	Day 4	8.00 ± 1.00^{n}
	Day 7	5.90 ± 0.74^{o}
	Day 10	5.90 ± 0.87^{o}
Overall acceptability	Day 1	7.60 ± 1.07^{pq}
	Day 4	8.27 ± 0.78^{p}
	Day 7	7.10 ± 0.73^{q}
	Day 10	5.90 ± 0.87^{r}

Conclusion

The successful preparation of oat milk yogurt using oat flakes and water in a 1:3 ratio; and 5% inoculum of *L. rhamnosus* and *L. paracasei* (1:1) resulted in a creamy, well-textured yogurt with desirable sensory qualities. Microbial analysis demonstrated a significant increase in lactobacillus counts during storage, with no contamination of fungus or coliforms detected. The gradual decrease in pH and sensory ratings over the 10-day refrigerated storage period is typical of probiotic yogurt fermentation, with the best sensory qualities observed on day 4.

Overall, this study demonstrated the potential for producing a stable, non-dairy oat yogurt enriched with *L. rhamnosus* and *L. paracasei* with good probiotic viability and acceptable sensory attributes.

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