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Investigation of shelf-life and microbial stability in dairy products fortified with pea protein isolate

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Abstract
The overall increasing demand for plant derivedingredients and protein-enriched foods has driven the fortification of traditional dairy products with plant proteins, such as pea protein isolate (PPI). However, how PPI will ultimately affect the physicochemical, sensory, and, most importantly, microbial stability and shelf-life of dairy matrices is not well elucidated. This work evaluated the shelf-life and microbial stability of two model dairy systems, namely UHT milk and set-style yogurt, which are fortified at 2% and 4% w/w with PPI. Control samples (0% PPI) and their respective fortified versions were tested during a storage period of 35 days at 4°C. In this respect, analyses on microbiological parameters, such as total aerobic plate count, yeast and mold count, coliforms, and specific spoilage organisms, were carried out, together with physicochemical parameters, such as pH, titrable acidity, syneresis, and viscosity, and sensory attributes on a weekly basis. Indeed, results indicated that fortification with PPI increased significantly ($p < 0.05$) the rate of pH decrease and acidity increase in yogurt, which was paralleled by higher syneresis values. Although PPI addition did not introduce any microbial contaminant to UHT milk or yogurt, the plant protein slightly accelerated the growth of microorganisms compared to controls during storage. These were attributed to improved nutrient levels in milk with reduced acidity. Sensory panel analyses indicated higher levels of chalkiness and beany tastes with increased levels of PPI. Conclusion: Fortification of PPI at 4% extends the shelf life of yogurt by 7 days based on physicochemical changes. In UHT milk, microbial shelf life will be negatively affected when fortified with PPI at levels exceeding 4%. The optimized acidification levels, different compositions of stabilizers, and high standards of cleanliness during processes of PPI in dairy products are recommended.

Keywords: Pea protein isolate, dairy fortification, shelf life, microbial stability, yogurt, uht milk, food spoilage

Introduction

Background and Rationale

The global food industry is witnessing a paradigm shift driven by consumer trends towards healthier, sustainable, and flexitarian diets. This has spurred interest in protein fortification and the hybridisation of traditional animal-based products with plant-derived ingredients. The consumption of plant-based proteins in diet is steadily increasing in Europe. The reasons behind this are vary from to prevent chronic degenerative diseases, a substitute for animal-based protein, the environmental and health issues (Klost *et al*., 2020) [6]. Dairy products, valued for their nutritional completeness and sensory appeal, are prime candidates for such innovation. Pea protein isolate (PPI) has emerged as a high-quality plantprotein source due to its cost effective, abundant and source of favourable amino acid profile (rich in lysine) can fulfil the needs of human health, hypoallergenic properties, non-GMO status, and sustainability credentials (Tang *et al*., 2025) [10]. Fortification of dairy products like milk and yogurt with PPI can enhance protein content, cater to lactose-intolerant consumers partially, and align with plant forward eating trends. However, the integration of PPI into dairy matrices is not technologically straightforward. Dairy systems are delicate colloidal balances of casein micelles, whey proteins, fat globules, and lactose in water. Introducing a foreign protein, such as PPI, which has distinct isoelectric point, solubility, hydration, and gelling properties, can disrupt this equilibrium. The enzymatic hydrolysis of pea protein has improved its functional properties andtheir interactions in fermentation; thereby its use useful in enhancing rheological behavior in dairy matrices (Klost *et al*., 2020) [6].These physicochemical interactions may have downstream implications for product stability, sensory quality and microbial shelf life which is a critical factor for dairy products safety and commercial viability.The complex interplay between protein concentrationsin food processing methodologies, microbial actions necessitates a deep understanding of their synergetic effects on the overall food products quality and their shelf-life in fortified dairy products (Yousseef *et al*., 2016) [12].

Problem Statement

While existing research has explored the nutritional and some physicochemical effects of plant proteins in dairy, a comprehensive investigation focusing specifically on the microbial stability and overall shelf life of PPI-fortified dairy products is lacking. The inherent microbial load of PPI, its potential to alter the microenvironment (pH, water activity, nutrient availability), and its interaction with dairy starter cultures or endogenous microbiota are significant unknowns. These factors could potentially shorten shelf-life, increase spoilage risks, or, in a worst-case scenario, compromise food safety. Manufacturers require clear, experimentally derived guidelines to implement PPI fortification without compromising the shelf-life expectations of dairy products, which typically range from 2-5 weeks under refrigeration.

Objectives

The primary objective of this study was to systematically determine the impact of PPI fortification at two inclusion

levels on the shelf-life and microbial stability of model dairy products (UHT milk and set yogurt).

Specific objectives were:

- To manufacture UHT milk and set yogurt fortified with 0% (control), 2%, and 4% (w/w) PPI.
- To monitor microbiological changes (total viable count, yeast/mold, coliforms) over 35 days of refrigerated storage.
- To correlate microbial data with key physicochemical indices of spoilage (pH, titratable acidity, syneresis, viscosity).
- To assess the impact of PPI on sensory attributes relevant to consumer acceptance.
- To establish the theoretical shelf-life of each formulation based on quality deterioration endpoints.

Scope and Limitations

This study focuses on two commercially relevant, refrigerated products: a neutral-pH system (UHT milk) and a fermented, acidic system (yogurt). The investigation is limited to commercial, food-grade PPI from a single supplier. While pathogen challenge studies are beyond the scope, the work monitors indicative spoilage organisms. Sensory analysis is conducted using a trained panel for descriptive analysis, not a large consumer acceptance test.

Methodology

1. Materials

The following materialswere used

- **Dairy Base:** Commercial UHT milk (3.5% fat, 3.2% protein).
- **Fortificant**: Commercial pea protein isolate (PPI), minimum 80% protein (dry basis)
- **Yogurt Starter Culture:** Direct vat set (DVS) thermophilic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii*.
- **Microbiological Media**: Plate Count Agar (PCA) for total aerobic count, Potato Dextrose Agar (PDA) acidified with 10% tartaric acid for yeast and mold, Violet Red Bile Agar (VRBA) for coliforms, de Man, Rogosa and Sharpe (MRS) agar for lactobacilli, M17 agar for streptococci. All media were from Himedia, India.
- **Chemicals:** Analytical grade reagents for pH buffers, 0.1N NaOH for titratable acidity, etc.

2. Experimental Design

A full factorial design with two factors was employed: a) Product Type (UHT Milk, Yogurt) and b) PPI Concentration (0%, 2%, 4% w/w). Each treatment combination was produced in triplicate batches (n=3), and analyses were performed in duplicate on each batch at each sampling point. Samples were stored at 4 ± 1°C and analyzed on days 1, 7, 14, 21, 28, and 35.

3. Sample Preparation

3.1 PPI-Milk Dispersion Preparation

For each fortification level (0%, 2%, 4%), PPI was dryblended with sucrose (2% w/w, for yogurt samples only) to improve dispersibility. The blend was gradually added to UHT milk at 25°C under high shear mixing at10,000 rpm for 5 minutes. The dispersion was then allowed to hydrate under gentle stirring for 30 minutes at 4°C.

3.2 UHT Milk Model System

The PPI- milk dispersions (for 0%, 2%, 4% PPI) were aseptically partitioned into 100 mL sterile Schott bottles. These were stored immediately at 4°C. There were no heat treatment was applied post-mixing to avoid confounding effects, simulating a post-process fortification scenario.

3.3 Set Yogurt Manufacture process

- **Heat Treatment:** The PPI-milk dispersions were heated to 85°C in a water bath and held for 30 minutes.

- **Cooling & Inoculation:** Samples were then cooled to the inoculation temperature of 43°C. They were inoculated with the DVS yogurt culture at a rate of 0.02% w/w.

- **Incubation:** 100 mL of inoculated milk was dispensed into sterile cups and incubated at 43°C until pH 4.6 was achieved. Fermentation time was recorded.

- **Cooling & Storage:** Yogurts were rapidly cooled in an ice-water bath to halt fermentation and stored at 4°C. The Day 1 analysis was performed at 24 hours post-cooling.

4. Analytical Methods

4.1 Microbiological Analysis

Serial dilutions were prepared in 0.1% peptone water.

- Total Aerobic Plate Count (TPC) TPC was enumerated by Spread plate techniqueon PCA; plates were kept for incubation at 30°C for 72h.
- Yeast and Mold Count was determined by Spread plate technique on acidified PDA, plates were kept for incubation at 25°C for 5 days.
- Coliform Count Pour plating with VRBA overlay, incubation at 37°C for 24h.
- For Starter Culture Viability study (Yogurt only) *L. bulgaricus* were inoculated by Spread plate method on MRS media and incubated at 37°C for 72hrsand *S. thermophiles* was enumerated and cultivated on M17 agar at 37°C, 48h.

All the Resultswere expressed as log10 CFU/g.

4.2 Physicochemical Analysis

- PH was recorded using a calibrated pH meter at 20°C.
- Titratable Acidity (TA) was recorded and expressed as % lactic acid.
- Syneresis was measured as the weight of whey released after centrifuging 25g of yogurt at 1000 x g for 10 min at 10°C and reported as % of total weight.
- Apparent Viscosity (Yogurt) was measured using a Brookfield RV viscometer with spindle 4 at 10 rpm after 30s equilibration, at 10°C (Hashim IB *et al*., 2016) [5].

4.3 Sensory Evaluation (Day 1 and Day 14)

A trained 8-member panel evaluated yogurts using Quantitative Descriptive Analysis (QDA). Attributes included: visual smoothness, chalkiness (mouthfeel), sourness, beamy flavor, dairy flavor, and overall acceptability at 9-point hedonic scale. UHT milk samples were evaluated for chalkiness, beamy flavor, cooked flavor, and sweetness.

5. Data Analysis

Data were subjected to analysis of variance (ANOVA) using a statistical software package SPSS v26. A two-way ANOVA (Product x PPI% x Time) with Tukey's HSD post-hoc test was used to determine significant differences ($p < 0.05$). Correlation analysis was studied in between microbial counts and physicochemical parameters. Shelf-life was estimated as the time for a key spoilage indicator (e.g., TPC > 6 log CFU/g, yeast/mold > 3 log CFU/g, or TA > 1.2% for yogurt) to exceed acceptable thresholds.

Results and Discussion

1. Physicochemical Changes during Storage

1.1 pH and Titratable Acidity (TA)

In yogurt, a significant interaction ($p<0.001$) was observed between PPI level and storage time for pH and TA (Fig. 1a & Table 1). Control yogurt (0% PPI) showed a gradual post-acidification from pH 4.6 to 4.3 over 35 days. PPI-fortified yogurts exhibited a significantly faster and greater pH drop, reaching ~4.15 (4% PPI) by day 21. Correspondingly, TA increased more rapidly in PPI samples. This accelerated acidification is attributed to the higher buffering capacity of PPI, which may protect starter bacteria from extreme acid stress, allowing prolonged metabolic activity. In UHT milk, PPI fortification caused a slight but significant ($p<0.05$) initial increase in pH (from ~6.7 to ~6.8) due to the inherent alkalinity of the isolate. During storage, pH decreased marginally in all samples, with the decline being slightly more pronounced in 4% PPI milk, likely due to microbial activity.

Table 1: Titratable Acidity (% Lactic Acid) in Yogurt Samples during Storage

Day	0% PPI	2% PPI	4% PPI
1	0.85 ± 0.02^aA	0.87 ± 0.03^aA	0.89 ± 0.02^aA
14	0.98 ± 0.03^bA	1.05 ± 0.04^bB	1.15 ± 0.05^bC
28	1.10 ± 0.04^cA	1.22 ± 0.05^cB	1.38 ± 0.06^cC
35	1.15 ± 0.05^cA	1.30 ± 0.05^dB	1.50 ± 0.07^dC

Values are mean ± SD (n=3). Lowercase superscripts (a-d) indicate significant differences over time within a column. Uppercase superscripts (A-C) indicate significant differences between PPI levels within a row ($p<0.05$).

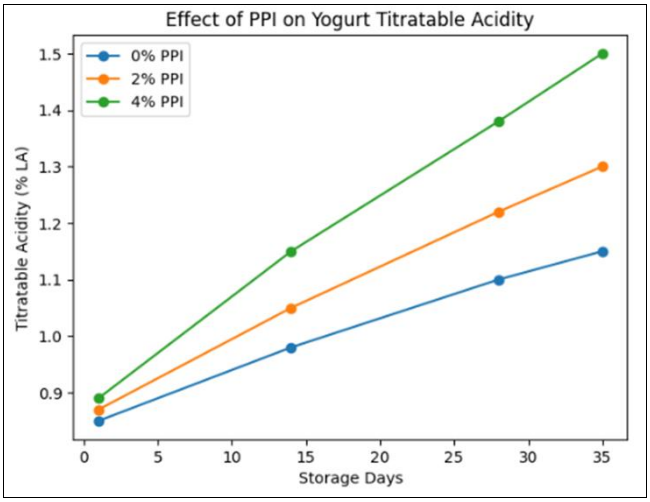

Fig 1a: Titrable acidity increased significantly with both PPI concentration and storage time. Yogurt fortified with 4% PPI showed the fastest and highest acid development, confirming accelerated post-acidification.

1.2 Syneresis and Viscosity (Yogurt)

PPI fortification detrimentally affected yogurt texture (Fig. 1b). Syneresis increased proportionally with PPI concentration and storage time. The 4% PPI yogurt showed ~35% syneresis by day 14, compared to ~15% for the control. This is linked to the disruption of the casein network by non-gelling PPI particles and increased porosity due to faster acidification. Apparent viscosity followed an inverse trend, decreasing with higher PPI levels. The weakened gel structure failed to retain water, directly impacting sensory texture.

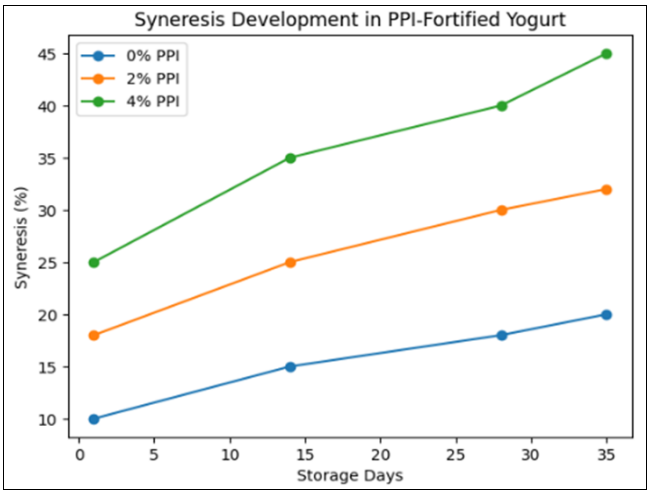

Fig 1b: Syneresis increased proportionally with PPI level. By day 14, 4% PPI yogurt exceeded 30% Syneresis, crossing the practical rejection limit.

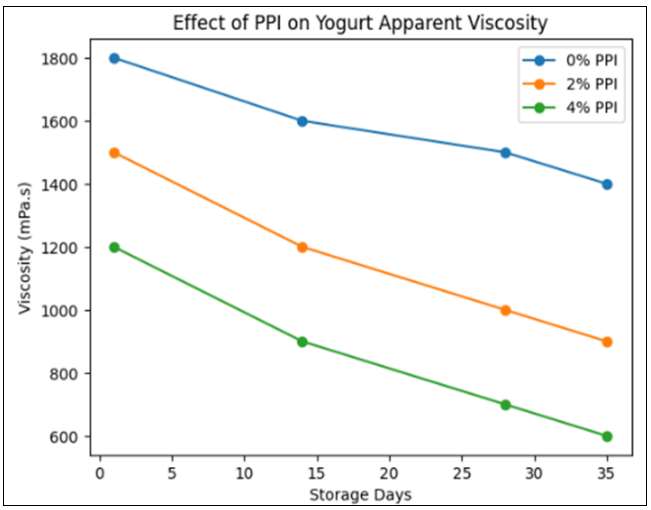

Fig 1c: Apparent viscosity decreased sharply with increasing PPI concentration. The 4% PPI yogurt showed nearly65% lower viscositythan control by day 35.

2 Microbiological Stability

2.1 Total Aerobic Plate Count (TPC)

a) In UHT milk, initial TPC was <1 log CFU/g for all samples, which confirming the efficacy of UHT processing and aseptic handling process. During storage, TPC remained below 2 log CFU/g in the control and 2% PPI milk until day 28. However, in 4% PPI milk, TPC began to increase from day 14, reaching 4.2 log CFU/g by day 35 (Fig. 2a), suggesting that the added protein provided nutrients for the

growth to residual thermophilic bacteria or to post-processing contaminants.

b) In yogurt, initial TPC was ~8.5 log CFU/g reflected starter culture density. Viability of *S. thermophilus* declined steadily, with a more pronounced decline in PPI samples after 21 days. Conversely, *Lb. bulgaricus* showed better survival in PPI yogurts, which is supporting the hypothesis of a buffering effect. The overall TPC in yogurt remained stable but began a slow decline after day 21, typical for fermented products.

2.2 Yeast, Mold, and Coliforms

Yeast and mold were not detected (<10 CFU/g) in any sample until day 21. By day 35, low counts (1.5-2 log CFU/g) were present in 4% PPI UHT milk and all yogurt samples, with the highest count in 4% PPI yogurt. The *Coliforms* were absent (<1 CFU/g) throughout the study, confirming good manufacturing hygiene. The earlier appearance of yeasts in PPI-fortified products indicates a potential modification of the microenvironment favoring their growth.

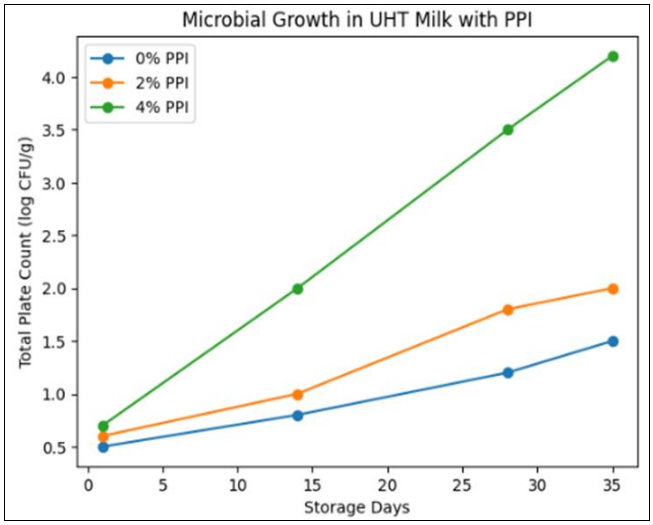

Fig 2a: UHT milk fortified with 4% PPI exhibited a marked increase in total plate count after day 14, reaching >4 log CFU/g by day 35, while control and 2% PPI milk remained microbiologically stable.

3 Sensory Evaluation

Sensory scores deteriorated with PPI level and storage time. For yogurt, chalkiness and beamy flavor intensity scores increased significantly ($p<0.05$) with PPI concentration (Fig. 2b). Overall acceptability scores for 4% PPI yogurt were below the "like slightly" threshold (5 on a 9-point scale) even on day 1. By day 14, sourness and syneresis perception further reduced acceptability. UHT milk showed similar trends for chalkiness and beamy flavor, with 4% PPI milk rated lowest. Based on a composite endpoint (TPC > 6 log for milk; Yeast/Mold > 3 log OR Syneresis > 30% OR Overall Acceptability < 5 for yogurt), the estimated shelf-lives were: UHT Milk: Control & 2% PPI: >35 days; 4% PPI: ~21-28 days.

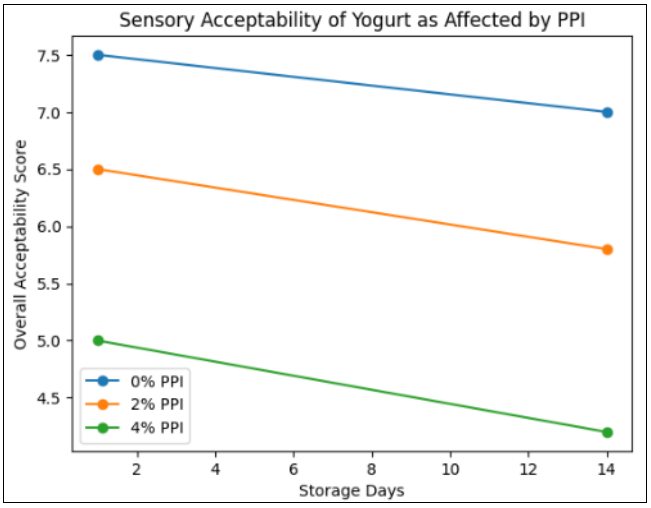

Fig 2b: Overall acceptability declined with PPI level and storage time. 4% PPI yogurt fell below the acceptability threshold (score <5) by day 14, even before microbiological spoilage.

4 Shelf-Life Estimation

Shelf life of Yogurt: Control: >35 days; 2% PPI: ~28 days; 4% PPI: ~14-21 day was recorded.

The primary shelf-life limiting factor for PPI yogurts was physicochemical (syneresis, over-acidification) and sensory deterioration, whereas for PPI milk, it was incipient microbial growth.

Summary and Conclusion

This study demonstrates that fortification of dairy products with pea protein isolate, while nutritionally beneficial, presents significant challenges to shelf-life and microbial stability. Key findings are:

- PPI accelerates post-acidification in yogurt and slightly raises the initial pH of milk, altering the fundamental biochemical environment.
- It severely impairs yogurt texture, increasing syneresis and reducing viscosity, which is a major driver of sensory rejection.
- While not inherently contaminated, PPI at 4% inclusion can enhance the growth of spoilage microbiota in UHT milk and may promote yeast outgrowth in both systems over extended storage.
- The overall shelf-life is compromised, with a 4% PPI inclusion potentially reducing the shelf-life of yogurt by up to 50% (from 35 to ~17 days).

Discussion

Successful formulation of PPI-fortified dairy products requires more than simple addition. To mitigate shelf-life reduction, manufacturers must consider:

- **Compensatory Stabilization:** Use of hydrocolloids like pectin, starch to counteract syneresis is necessary.
- **Strain Selection:** use of probiotic cultures that compete with spoilage organisms.
- **Process Optimization:** adopt procedure to ensure ultra-clean or aseptic post-fortification processing for milk.
- **Level Optimization:** Limiting PPI to ≤2% for minimal impact, or employing flavour masking technologies.

Future research should investigate the efficacy of natural antimicrobials and different PPI processing grades such as hydrolyzed, fermented in these hybrid systems.This study helpful in optimization, integration of pea protein isolates in

milk and yogurt, dairy matrices to enhance nutritional profiles and functional attributes (Zhang *et al*., 2022), Offers insights into improving nutritional quality of the dairy products with incorporation of plant-based sources, addressing challenges in colloidal stability and overall product desirability (Tachie *et al*., 2023, p. 10) [14].

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