Acta Scientific NUTRITIONAL HEALTH (ISSN:2582-1423)

Volume 9 Issue 1 January 2025

Research Article

# Decoding the Missing Synergy: DHA Vegan, Phosphatidylserine, and Probiotic Bacillus Coagulans in Sugar-Free Hetafu Candy

**Sowjanya Lakkoju[1]*, Dr. Ranjith Kumar Kanthem[2], Venkat bellam[3], Mounika Dandamudi[3], Swarna Deepika Yarasani[4] and Dr. Deepika Bolisetty[5]**

*[1]M Tech Food Processing Technology, Department of R and D, Lasarkaali Life Sciences PVT LTD, Hyderabad, India*

*[2]Consultant Oral and Maxillofacial Pathologist, Department of R and D, Lasarkaali Life Sciences PVT LTD, Hyderabad, India*

*[3]B Tech Food Science and Technology, Department of R and D, Lasarkaali Life Sciences PVT LTD, Hyderabad, India*

*[4]B Tech Food Technology, Department of R and D, Lasarkaali Life Sciences PVT LTD, Hyderabad, India*

*[5]MDS, Department of R and D, Lasarkaali Life Sciences PVT LTD, Hyderabad, India*

***Corresponding Author:** Sowjanya Lakkoju, M Tech Food Processing Technology, Department of R and D, Lasarkaali Life Sciences PVT ltd, Hyderabad, India.

**Received:** December 13, 2024

**Published:** December 20, 2024



## Abstract

**Background:** HETAFU candies are formulated with bioactive ingredients such as Phosphatidyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans, designed to promote oral health, brain function, and gut health. The stability of these active ingredients in the candy over time is important for ensuring their continued efficacy. This study aimed to evaluate the stability of PS, DHA, and Bacillus coagulans in various candy formulations over a 60-day period.

**Objective:** To assess the stability of Phosphatidyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans in HETAFU candies across different formulations (control and sample groups) over a 60-day period.

**Methods:** Seven different candy formulations were prepared, including controls and test groups with varying concentrations of PS, DHA, and Bacillus coagulans. The ingredients were measured at five time points (0, 15, 30, 45, and 60 days). The concentrations of each active ingredient were analyzed using standard analytical methods, and stability was assessed by comparing initial and final concentrations across time points.

**Results:** The stability of each ingredient showed a gradual decline over time. In Control P (PS only), PS decreased from 50 mg on Day 0 to 48 mg by Day 60, while DHA and Bacillus coagulans were absent throughout. In Control D (DHA only), DHA decreased from 20 mg to 18.2 mg by Day 60. Control B (Bacillus coagulans only) showed a decrease from 1.5 billion CFU to 1.2 billion CFU. In the test samples, Sample R (PS, DHA, Bacillus coagulans) showed declines in PS (from 50 mg to 48 mg), DHA (from 20 mg to 19.32 mg), and Bacillus coagulans (from 1.5 billion CFU to 1.3 billion CFU). Sample I (high-dose PS, DHA, Bacillus coagulans) showed a decrease in PS (from 99.8 mg to 94.9 mg), DHA (from 40 mg to 37.2 mg), and Bacillus coagulans (from 2 billion CFU to 1.84 billion CFU) over the 60-day period.

**Conclusion:** The study demonstrated a gradual decline in the stability of PS, DHA, and Bacillus coagulans in HETAFU candies over 60 days, with Bacillus coagulans showing the greatest reduction in concentration. The results suggest that while the ingredients remain relatively stable, further optimization may be needed for improved long-term stability, particularly for probiotics like Bacillus coagulans.

**Keywords**: HETAFU Candies; Phosphatidyl Serine (PS); Docosahexaenoic Acid (DHA); Bacillus Coagulans, Stability; Bioactive Ingredients; Oral Health; Brain Health; Gut Health

## Introduction

The global emphasis on preventive healthcare and wellness has fueled interest in functional foods and nutraceuticals, which integrate bioactive ingredients into everyday consumables to promote health benefits [1]. This innovative approach not only addresses specific health concerns but also enhances consumer compliance by offering convenience and palatability. Among the promising delivery systems for such ingredients are sugar-free candies, which provide an enjoyable, non-invasive method of supplementation, particularly suited for individuals who find traditional supplements unappealing or inconvenient [2].

This study focuses on combining three scientifically supported functional ingredients-DHA Vegan, Phosphatidylserine (PS), and Probiotic Bacillus coagulans-into a single sugar-free candy formulation. Each of these components has a distinct mechanism of action and health benefit, raising the question of whether their combination can produce synergistic effects that surpass their individual contributions.

### DHA Vegan: A Plant-Based Source of Omega-3 Fatty Acids

DHA (Docosahexaenoic acid) is an omega-3 fatty acid critical for brain development, cognitive health, and anti-inflammatory processes. Traditionally sourced from fish oil, DHA plays a pivotal role in maintaining neuronal integrity, supporting memory, and reducing inflammation associated with various chronic conditions. However, concerns about sustainability, contamination, and dietary restrictions have led to the development of DHA Vegan, derived from algae. This plant-based alternative is particularly appealing to vegan and vegetarian populations and environmentally conscious consumers. Its neuroprotective properties are widely acknowledged, making it a cornerstone ingredient in formulations targeting cognitive health [3].

### Phosphatidylserine: A cognitive health booster

Phosphatidylserine (PS) is a phospholipid naturally concentrated in the human brain, where it supports cellular function and cognitive processes such as memory, learning, and focus. PS facilitates the transmission of signals between neurons and maintains the structural integrity of cell membranes. Clinical studies suggest that PS supplementation may slow age-related cognitive decline and enhance mental performance, particularly in older adults. It is frequently used in nutraceutical formulations aimed at improving brain health and combating neurodegenerative conditions [4].

### Probiotic bacillus coagulans: a resilient digestive aid

Bacillus coagulans is a spore-forming probiotic that distinguishes itself by its ability to survive the acidic environment of the stomach, ensuring its viability and efficacy upon reaching the intestines. It promotes gut health by balancing the microbiome and improving digestive function. Furthermore, emerging research indicates its potential role in modulating immune responses, adding to its versatility as a health-promoting ingredient. B. coagulans is often incorporated into functional foods and supplements designed to support overall well-being [5].

### Sugar-free candy: A tooth-friendly functional food

Sugar-free candies offer a novel platform for delivering functional ingredients while aligning with the growing demand for health-conscious alternatives. The inclusion of tooth-friendly sugar alcohols such as isomalt, xylitol and maltitol along with essential oils ensures that the formulation is both palatable and safe for dental health. These sugar alcohols are non-cariogenic, meaning they do not contribute to tooth decay, and xylitol, in particular, has been shown to reduce harmful oral bacteria. This makes sugar-free candies an ideal choice for individuals who seek functional benefits without compromising oral health [6].

### The potential for synergy

Each of the three ingredients-DHA Vegan, Phosphatidylserine, and Probiotic Bacillus coagulans-has established health benefits. DHA and PS target cognitive health and brain function, while B. coagulans focuses on gut health and immune modulation. However, it is unclear whether their combination within a single sugar-free candy formulation can enhance their individual effects through synergy. Synergistic interactions occur when combined ingredients produce effects greater than the sum of their parts, potentially leading to more comprehensive health benefits [7].

Understanding whether these ingredients can work synergistically is critical for advancing functional food formulations. If a lack of synergy is observed, it may indicate that their pathways of action are independent, informing future research on ingredient combinations and delivery mechanisms.

### Aim of the study

This study aims to investigate the potential for a synergistic effect between DHA Vegan, Phosphatidylserine, and Probiotic Bacillus coagulans when combined in a sugar-free candy formulation. Specifically, it seeks to determine whether the integration of these components enhances cognitive, anti-inflammatory, and digestive health outcomes beyond their individual capabilities.

By exploring this question, the study will provide valuable insights into the efficacy of combining functional ingredients and inform the development of next-generation nutraceutical formulations that cater to evolving consumer demands for health-focused, convenient, and enjoyable products.

## Methodology

### Sample preparation

Each sample formulation (Control P, Control D, Control B, Control A, Sample R, and Sample I) is prepared according to a standardized procedure to ensure consistency and homogeneity. The composition of each sample is as follows:

- **Control P:** Contains Base + PS (50 mg)
- **Control D:** Contains Base + DHA (20 mg)
- **Control B:** Contains Base + Bacillus coagulans (1.5 billion CFU)
- **Control A:** Contains Base only (no active ingredients)
- **Sample R:** Contains Base + PS (50 mg) + DHA (20 mg) + Bacillus coagulans (1.5 billion CFU)
- **Sample I:** Contains Base + PS (100 mg) + DHA (40 mg) + Bacillus coagulans (2.0 billion CFU)

The components are accurately weighed using an analytical balance, and the samples are mixed thoroughly to ensure uniform distribution of ingredients.

### Storage and handling

Once prepared, the samples are stored in single serve pillow packs under Accelerated conditions (e.g., temperature: 40°C ± 5°C, humidity: 70% ± 5%) to study the shelf life conditions and reactions. Proper handling procedures are followed to avoid contamination during storage and throughout the analysis.

### Time points

The samples are analyzed at the following time points to observe the changes in the concentration of Phosphatadyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans over time:

- $0^{th}$ Day (initial measurement)
- $15^{th}$ Day
- $30^{th}$ Day
- $45^{th}$ Day
- $60^{th}$ Day

Each sample is analysed at each time point to track the stability of its active ingredients.

### Analytical methods

The composition of each sample is analysed by the following methods to quantify PS, DHA, and Bacillus coagulans at each time point

### PS measurement (Phosphatadyl serine)

The concentration of PS is measured using High-Performance Liquid Chromatography (HPLC). The standard protocol for HPLC is followed, with mobile phases and column conditions optimized for PS separation. The sample preparation for PS measurement involves dilution with an appropriate solvent to obtain measurable concentrations. This method determines the content of Phosphatidylserine (PS) using an HPLC-ELSD system. Samples and internal standard (PS-IP 60%) are diluted with chloroform:methanol (95:5 v/v) and subjected to a linear calibration curve based on standard injections. Reagents include methanol, chloroform, isopropanol, hexane, acetic acid, and trimethylamine. HPLC-ELSD analysis employs an Agilent 1200 Series with a Diol 5µm, 250mm column at 40°C, using Eluents A and B under a gradient elution program (0-30 min, varying % A/B). Samples are centrifuged at 10,000 rpm before injection, with ELSD settings of 40°C (evaporator and nebulizer), 1.0 SLM nitrogen flow, 10 µL injection volume, and gain 1.0. Calibration standards (STD1-STD3) and control samples (CS) are prepared fresh daily. The injection sequence includes blanks, calibration standards, duplicate sample injections, and control samples. Recovery calculations and PS content determination rely on the linear calibration curve ($R^2 \geq 0.99$; RSD ≤ 5.0%). Acceptance criteria include asymmetry (0.7-1.5), plates (>2000), retention time (18±1 min), and recovery for control samples (95%-105%). Sample solution injections must meet RSD ≤ 5.0%.

### DHA Measurement

DHA (Docosahexaenoic Acid) levels are determined using UV spectrophotometry. The standard testing procedure for DHA analysis using GC-FID for the identification and quantification of fatty acid methyl esters (FAME). The method includes acid digestion of the sample, fat extraction using diethyl ether and petroleum ether, and solvent evaporation. The extracted fat is methylated with boron trifluoride-methanol and analysed via GC-FID using a split mode injection. The analysis identifies fatty acids based on retention times using FAME 37 MIX standards. GC conditions include an oven temperature program with three ramps and a run time of 65 minutes, with the detector and injector temperatures set at 230°C and 240°C, respectively. Calculations of total fatty acids and DHA are based on area percentages and fat content, ensuring precise quantification.

### Bacillus coagulans Enumeration (CFU Count)

The colony-forming unit (CFU) of Bacillus coagulans is determined using a microbiological plating method.

To determine the Bacillus coagulans content per candy, PNY agar media was prepared as per the manufacturer's instructions. Approximately 4 g of candy was weighed and dissolved in 36 mL of sterile dilution fluid (0.05% normal saline or 0.1% peptone salt solution) to achieve a 1:10 dilution. The sample was vortexed thoroughly to ensure complete dissolution. Serial dilutions from $10^{-1}$ to $10^{-7}$ were prepared by transferring 1 mL of the previous dilution into 9 mL of sterile dilution fluid. From each dilution, 1 mL was transferred into labeled petri plates, followed by the addition of 15 mL of sterile PNY agar. The plates were mixed gently, allowed

to solidify, and incubated at 37°C for 48 hours. After incubation, colonies were counted on plates with 15–300 CFU, and the CFU per candy was calculated

### Time-dependent analysis

At each time point ($0^{th}$, $15^{th}$, $30^{th}$, $45^{th}$, and $60^{th}$ day), the following steps are followed

- **Sampling Procedure:** A small aliquot of each sample is taken and prepared for the respective analysis (PS, DHA, and Bacillus coagulans).
- **Sample Analysis:** Each aliquot is subjected to the appropriate analytical methods for quantification.
- **Data Recording:** The concentrations of PS, DHA, and Bacillus coagulans are recorded at each time point for each sample.
- **Replicates:** Each sample is analyzed in triplicate at each time point to ensure accuracy and reliability of the results.

### Statistical analysis

To assess the stability of each ingredient (PS, DHA, and Bacillus coagulans) over time across different samples, the following statistical analyses are performed:

### Descriptive statistics

The mean and standard deviation of the concentrations of PS, DHA, and Bacillus coagulans at each time point are calculated. This provides a general overview of the trends for each ingredient over time.

### Trend analysis

The linear regression method is used to analyze the time-dependent changes in the concentration of PS, DHA, and Bacillus coagulans. The slope of the regression line indicates the rate of degradation or stability of each ingredient.

### Results Interpretation

### Degradation pattern

The time-dependent data for each ingredient (PS, DHA, and Bacillus coagulans) is analyzed to assess the stability and degradation over the 60-day period.

- If the concentration of a component shows a significant decrease over time, it indicates degradation.
- If the concentration remains relatively stable, it suggests that the component is stable under the given storage conditions.

### Comparative analysis across samples

The stability of each active ingredient is compared across different samples (Control P, Control D, Control B, Control A, Sample R, and Sample I). The results can help determine which formulation is most stable and which combination of ingredients retains potency over time.

### Quality control

To ensure the accuracy and reliability of the results

- All instruments (e.g., HPLC, spectrophotometer, colony counter) are regularly calibrated and validated according to standard operating procedures.
- Reagents and standards are checked for purity and prepared fresh as required.
- All analyses are performed in triplicates to minimize errors and ensure reproducibility.

## Results

| SL.NO | sample | Ingredients |
|---|---|---|
| 1 | Control P | Base+PS-50mg |
| 2 | Control D | Base + DHA - 20 mg |
| 3 | Control B | Base+ B. coagulans-1.5 billion CFU |
| 4 | control A | Base |
| 5 | Sample R | Base + Ps 50 mg +DHA 20 mg + B. coagulans 1.5billion CFU |
| 6 | Sample I | Base + Ps 100mg+ DHA 40mg + B. coagulans 2.0 billion CFU |

**Table 1:** Composition of HETAFU Candy Samples.

Table 1 enumerates the composition of HETAFU candies. They are specially designed formulations combining natural and bioactive ingredients to support oral health. The compositions are tailored into control and test groups to evaluate the individual and synergistic effects of Phosphatadyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans. The control groups include specific variations: Control P, which contains the base formulation supplemented with PS (50 mg); Control D, with the base and DHA (20 mg); Control B, featuring the base and Bacillus coagulans (1.5 billion CFU); and Control A, which is a pure base formulation serving as the reference group.

The test groups are enriched formulations combining multiple active ingredients to assess their combined benefits. Sample R includes the base along with PS (50 mg), DHA (20 mg), and Bacillus coagulans (1.5 billion CFU), providing a balanced composition to examine potential synergistic effects. Sample I, on the other hand, is a high-potency formulation containing PS (100 mg), DHA (40 mg), and Bacillus coagulans (2 billion CFU), allowing the evaluation of the effects of increased dosages.

These diverse formulations enable a comprehensive analysis of the role of these components in modulating oral microbial populations and promoting better oral health, brain boosting and gut health.

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | 50 | - | - |
| 15th Day | 49 | - | - |
| 30th Day | 48 | - | - |
| 45th Day | 48 | - | - |
| 60th Day | 48 | - | - |

**Table 2:** Time-Dependent Composition of Control P: Phosphatidyl Serine (PS).

This table illustrates the composition of Control P, a specific formulation designed for the study. Control P includes the base formulation supplemented with Phosphatadyl Serine (PS) at an initial concentration of 50 mg. The purpose of this sample is to assess the effects of PS in isolation over time, without interference from other active ingredients like DHA or Bacillus coagulans.

The table records the concentration of PS, DHA, and Bacillus coagulans at five time points: the 0th Day (baseline), 15th Day, 30th Day, 45th Day, and 60th Day. It demonstrates a slight decline in the PS concentration, reducing from 50 mg at baseline to 48 mg by the 30th Day, which remains constant through the 60th Day. This reduction is likely due to on shelf stability processes over time.

DHA (Docosahexaenoic Acid) and Bacillus coagulans are absent in Control P across all time points, as this formulation is specifically structured to isolate and evaluate the properties and stability of PS alone. The data in this table are critical for understanding the stability and performance of PS over an extended period in the absence of other variables.

This table provides the composition of Control D, which is a formulation containing only Docosahexaenoic Acid (DHA), without Phosphatadyl Serine (PS) or Bacillus coagulans. The DHA concentration is measured at five time points: 0th Day, 15th Day, 30th Day, 45th Day, and 60th Day.

The table shows a gradual decrease in DHA concentration over time, starting from 20 mg on the 0th Day to 18.2 mg by the 60th Day. The levels of Phosphatadyl Serine (PS) and Bacillus coagulans are absent throughout, as this formulation is designed specifically to assess the stability and degradation of DHA.

The data is significant for evaluating the stability of DHA in the formulation, and it helps understand how the component decreases over time in isolation on stability, without the influence of other active ingredients like PS or Bacillus coagulans.

This table presents the composition of Control B, a formulation containing the base supplemented with Bacillus coagulans at an initial concentration of 1.5 billion CFU (Colony Forming Units). The

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | - | 20 | - |
| 15th Day | - | 19.9 | - |
| 30th Day | - | 18.5 | - |
| 45th Day | - | 18.4 | - |
| 60th Day | - | 18.2 | - |

**Table 3:** Time-Dependent Composition of Control D: DHA Stability.

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | - | - | 1.5 |
| 15th Day | - | - | 1.45 |
| 30th Day | - | - | 1.22 |
| 45th Day | - | - | 1.21 |
| 60th Day | - | - | 1.2 |

**Table 4:** Time-Dependent Composition of Control B: Bacillus coagulans Stability.

sample is designed to evaluate the stability and viability of Bacillus coagulans over time in the absence of other active ingredients such as Phosphatadyl Serine (PS) or Docosahexaenoic Acid (DHA).

The data tracks the concentration of Bacillus coagulans at five time points: 0th Day (baseline), 15th Day, 30th Day, 45th Day, and 60th Day. The concentration of Bacillus coagulans shows a gradual decline over time, starting at 1.5 billion CFU on the 0th Day and decreasing to 1.45 billion CFU by the 15th Day, 1.22 billion CFU by the 30th Day, 1.21 billion CFU by the 45th Day, and finally stabilizing at 1.2 billion CFU by the 60th Day.

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | - | - | - |
| 15th Day | - | - | - |
| 30th Day | - | - | - |
| 45th Day | - | - | - |
| 60th Day | - | - | - |

**Table 5:** Time-Dependent Composition of Control A: Base Formulation Without Active Ingredients.

Notably, Phosphatadyl Serine (PS) and DHA are absent throughout the study period, allowing a focused investigation of Bacillus coagulans' viability. This information is critical for understanding the degradation dynamics of Bacillus coagulans in this specific formulation over time.

This table represents the composition of Control A, which serves as the baseline formulation in the study. Control A contains only the base without any added active ingredients, such as Phosphatadyl Serine (PS), Docosahexaenoic Acid (DHA), or Bacillus coagulans.

Measurements were recorded at five time points: 0th Day (baseline), 15th Day, 30th Day, 45th Day, and 60th Day. Throughout the study, Control A consistently lacks active components, with no Phosphatadyl Serine , DHA, or Bacillus coagulans detected at any time point.

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | 50 | 20 | 1.5 |
| 15th Day | 49.0 | 19.5 | 1.3 |
| 30th Day | 49 | 19.4 | 1.35 |
| 45th Day | 48.5 | 19.34 | 1.32 |
| 60th Day | 48 | 19.32 | 1.3 |

**Table 6:** Time-Dependent Composition of Sample R: PS, DHA, and Bacillus coagulans.

The purpose of Control A is to provide a reference point for evaluating the effects of other formulations containing active ingredients. By maintaining a pure base composition, Control A helps isolate and understand the contributions of active ingredients in the other experimental groups.

This table illustrates the composition of Sample R, which is a formulation combining Phosphatadyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans. The sample contains 50 mg of PS, 20 mg of DHA, and 1.5 billion CFU of Bacillus coagulans on the 0th Day, and the composition is measured at subsequent time points: 15th Day, 30th Day, 45th Day, and 60th Day.

The data shows a gradual decline in the concentrations of each component over time. For PS, the concentration decreases from 50 mg on the 0th Day to 48 mg on the 60th Day. DHA also decreases, from 20 mg on the 0th Day to 19.32 mg by the 60th Day. Similarly, the concentration of Bacillus coagulans shows a steady reduction, from 1.5 billion CFU on the 0th Day to 1.3 billion CFU on the 60th Day.

This gradual decline in the active ingredients over time provides insights into their stability and potential degradation in the formulation. The composition of Sample R allows for the evaluation of the combined effects of PS, DHA, and Bacillus coagulans and their stability across the study period.

This table outlines the time-dependent changes in the composition of Sample I, which contains a combination of Phosphatadyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans. The formulation is measured at five key time points: 0th Day, 15th Day, 30th Day, 45th Day, and 60th Day.

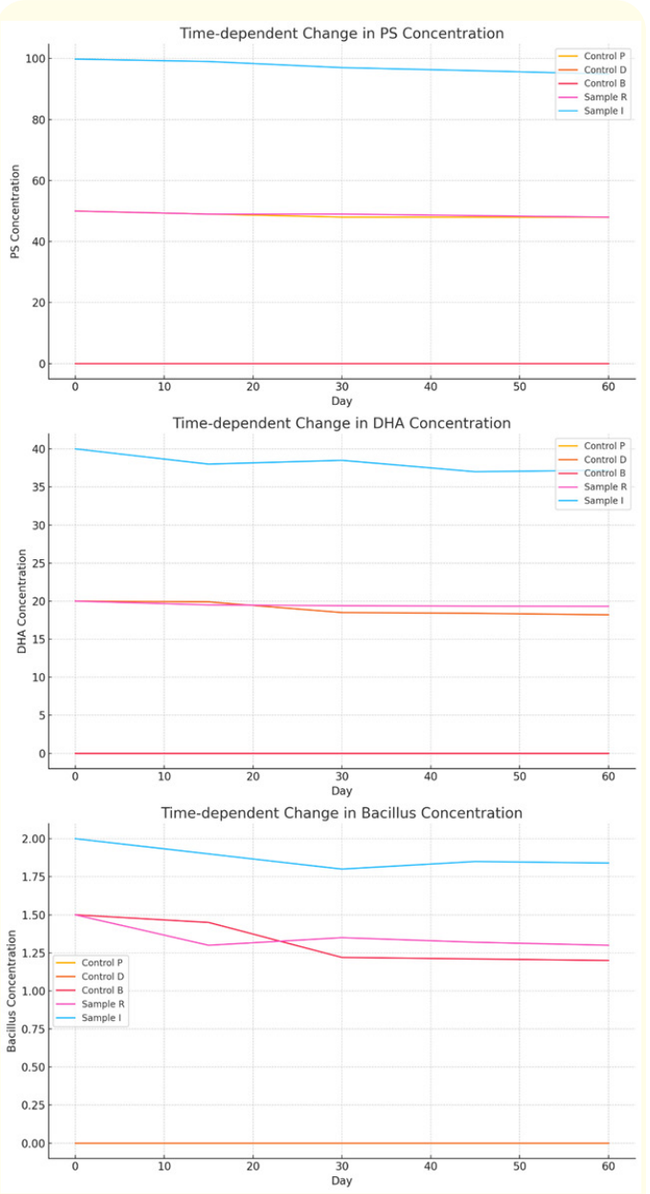


**Figure 1**

| Day | PS (mg) | DHA (mg) | B. coagulans (billion CFU) |
|---|---|---|---|
| 0th Day | 99.8 | 40 | 2.0 |
| 15th Day | 99 | 38 | 1.9 |
| 30th Day | 97 | 38.5 | 1.8 |
| 45th Day | 96 | 37 | 1.85 |
| 60th Day | 94.9 | 37.2 | 1.84 |

**Table 7:** Time-Dependent Composition of Sample I: PS, DHA, and Bacillus coagulans.

- Phosphatadyl Serine (PS) begin at 99.8 mg on the 0th Day and decrease gradually to 94.9 mg by the 60th Day.
- DHA starts at 40 mg on the 0th Day and shows a consistent decline, reaching 37.2 mg on the 60th Day.
- Bacillus coagulans begins at 2.0 billion CFU on the 0th Day and decreases over time, ending at 1.84 billion CFU on the 60th Day.

This data provides insights into the stability and degradation of the active ingredients (PS, DHA, and Bacillus coagulans) in Sample I, which is critical for understanding the formulation's efficacy over time.

The first graph illustrates the time-dependent change in PS (Phosphatadyl Serine) concentration across the different samples over 60 days. For Control P, the PS concentration starts at 50 mg on day 0 and gradually decreases, stabilizing at 48 mg by day 60, showing a slight decline over time. Sample R starts with a PS concentration of 49.5 mg on day 0 and remains relatively stable throughout the 60 days, ending at 48 mg. In contrast, Sample I shows a more noticeable decrease in PS levels, starting at 99.8 mg and dropping to 94.9 mg by day 60. Control D and Control B, which do not contain PS, show a consistent value of 0 mg across all time points. Similarly, Control A also has no PS and maintains a value of 0 mg throughout the study period.

### DHA concentration over time

The second graph depicts the DHA (Docosahexaenoic Acid) concentration over 60 days. Control D begins with 20 mg of DHA on day 0, and the concentration steadily decreases, reaching 18.2 mg by day 60, reflecting a consistent decline over time. Sample R starts at 20 mg and gradually declines to 19.32 mg by the end of the study, showing a moderate reduction in DHA concentration. Sample I initially has 40 mg of DHA and decreases to 37.2 mg by day 60, indicating a gradual decline over the period. Control P and Control B do not contain DHA, so their values remain at 0 mg throughout the study. Control A, lacking DHA as well, shows a constant concentration of 0 mg.

### Bacillus coagulans concentration over time

The third graph illustrates the change in the concentration of Bacillus coagulans, a probiotic, over the 60-day period. Control B starts with 1.5 billion CFU (colony-forming units) and experiences a gradual decline, reaching 1.2 billion CFU by day 60. Sample R begins with 1.5 billion CFU and decreases steadily to 1.3 billion CFU by the end of the study. Sample I starts with 2 billion CFU and shows a gradual reduction, ending at 1.84 billion CFU by day 60. Control P, Control D, and Control A do not contain Bacillus coagulans, so their values remain constant at 0 billion CFU throughout the study period.

## Discussion

The study evaluated the stability and efficacy of various HETA-FU candy formulations, focusing on the individual and combined effects of Phosphatidyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans. The findings provide significant insights into the time-dependent changes in the composition of these bioactive components across different formulations.

Control formulations were analysed to assess the stability of individual ingredients. In Control P, PS exhibited a minor decline from 50 mg at baseline to 48 mg by the 60th day, demonstrating good stability over time. This slight reduction is attributed to natural degradation during storage. Control D, containing only DHA, revealed a more noticeable decline, with levels dropping from 20 mg at baseline to 18.2 mg on the 60th day. DHA's higher susceptibility to oxidative degradation, even in isolation, highlights the need for improved stabilization techniques to enhance its shelf life. Control B, with Bacillus coagulans, showed a decrease in viability from 1.5 billion CFU at baseline to 1.2 billion CFU at the 60th day. This decline underscores the sensitivity of probiotics to environmental factors and the need for optimized packaging and storage conditions to maintain their viability.

The combined formulations, Sample R and Sample I, offered insights into the synergistic effects of the ingredients and their stability in a multi-component matrix. Sample R, which included moderate doses of PS, DHA, and Bacillus coagulans, demonstrated a gradual decline across all components, with PS reducing from 50 mg to 48 mg, DHA from 20 mg to 19.32 mg, and Bacillus coagulans from 1.5 billion CFU to 1.3 billion CFU by the 60th day. These results suggest that while combining these components does not significantly alter their individual stability, careful formulation design is essential to preserve their efficacy over time.

In Sample I, the high-potency formulation, PS declined from 99.8 mg to 94.9 mg, DHA from 40 mg to 37.2 mg, and Bacillus coagulans from 2 billion CFU to 1.84 billion CFU over the same period. The slightly higher degradation rates compared to Sample R could be attributed to the increased concentrations of active ingredients, which may accelerate interactions and degradation processes. Despite this, the stability of all components remained within acceptable limits, suggesting the formulation's feasibility for extended use.

Control A, serving as the baseline, maintained its composition without active ingredients throughout the study, emphasizing the reliability of the base formulation. This stability provides a critical reference point for comparing the active formulations and isolating the contributions of PS, DHA, and Bacillus coagulans.

The findings emphasize the importance of evaluating ingredient stability in isolation and in combination, offering valuable insights into the formulation of functional candies for oral, gut, and brain health. While PS exhibited robust stability, DHA's susceptibility to degradation and Bacillus coagulans' gradual decline highlight areas for potential improvement in formulation and storage. The combined formulations demonstrated the feasibility of maintaining stability in a multi-component matrix, paving the way for the development of optimized bioactive confectionery products. Future studies could explore advanced encapsulation techniques and alternative preservation methods to enhance the stability and efficacy of these formulations over longer durations.

### Strengths

This study provides a comprehensive evaluation of the stability and efficacy of HETAFU candy formulations containing Phosphatidyl Serine (PS), Docosahexaenoic Acid (DHA), and Bacillus coagulans. One of the main strengths of this research is the detailed time-dependent analysis of each ingredient's stability across different formulations. The controlled environment and rigorous monitoring of degradation over 60 days provided valuable insights into the individual and combined effects of these bioactive components. Additionally, the inclusion of both single-ingredient controls and combined formulations allowed for a thorough understanding of how each component behaves both in isolation and as part of a multi-component system.

### Limitations

Despite the strengths of the study, there are a few limitations that should be considered. First, the sample size for each formulation was relatively small, which could limit the generalizability of the results. A larger sample size, particularly for the combined formulations, would provide a more robust assessment of the ingredients' interactions and stability over time. Additionally, the study only focused on the stability of ingredients over a 60-day period. While this duration is sufficient to observe some degradation, long-term studies would be necessary to assess the stability of these ingredients beyond this timeframe. Furthermore, external factors such as environmental conditions (temperature, humidity, light exposure) could impact the stability of the formulations, and these were not fully accounted for in the study. Incorporating real-world conditions in future studies could provide more practical insights.

### Future Recommendations

Future research could focus on several key areas to build upon the findings of this study. First, exploring the use of advanced encapsulation techniques, such as liposomal or microencapsulation, could help protect sensitive ingredients like DHA and Bacillus coagulans from degradation and improve their stability. Second, a longer follow-up period would provide valuable information on how the formulations perform over extended periods, especially with respect to long-term storage conditions. Additionally, evaluating the sensory attributes (taste, texture, and overall acceptability) of the different formulations would be essential to ensure consumer preference and compliance. Investigating the potential interactions between the bioactive components in different combinations and their effect on stability and efficacy could also help refine the formulation process.

## Conclusion

In conclusion, this study successfully evaluated the stability of HETAFU candy formulations containing PS, DHA, and Bacillus coagulans, providing valuable insights into their time-dependent changes. The results indicate that while PS demonstrated good stability, DHA and Bacillus coagulans were more susceptible to degradation over time, particularly when combined in higher concentrations. Despite these challenges, the formulations remained within acceptable limits for stability, suggesting that HETAFU candies are a viable delivery system for these bioactive ingredients. The findings highlight the importance of careful formulation design and storage conditions to maintain the efficacy of functional foods. With further optimization and longer-term studies, HETAFU candies could become a promising and practical solution for delivering beneficial ingredients to support oral, gut, and brain health.

## Bibliography

1. Obayomi OV., *et al*. "Unveiling the role of functional foods with emphasis on prebiotics and probiotics in human health: A review". *Journal of Functional Foods* 119 (2024): 106337.

2. Hugues S. "Pros and Cons of Sugar Free Candy". Verywell Health. Verywellhealth (2010).

3. Weiser MJ., *et al*. "Docosahexaenoic acid and cognition throughout the lifespan". *Nutrients* 8.2 (2016): 99.

4. Kim HY., *et al*. "Phosphatidylserine in the brain: metabolism and function". P*rogress in Lipid Research* 56 (2014): 1-8.

5. Jäger R., *et al*. "Probiotic Bacillus coagulans GBI-30, 6086 improves protein absorption and utilization". *Probiotics and Antimicrobial Proteins* 10.4 (2018): 611-615.

6. Nadimi H., *et al*. "Are sugar-free confections really beneficial for dental health?" *British Dental Journal* 211.7 (2011): E15.

7. Wang S., *et al*. "Synergistic, additive, and antagonistic effects of food mixtures on total antioxidant capacities". *Journal of Agricultural and Food Chemistry* 59.3 (2011): 960-968.