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Development of novel non-dairy synbiotic chocolates for promoting desirable gut microbiome

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Abstract
This research focuses on the development and analysis (physico-chemical analysis and probiotic viability count) of non-dairy synbiotic chocolates, targeting consumers who are lactose intolerance, vegans, or seeking health-enhancing food options. The formulations were enriched with almond milk, oats powder, beetroot powder and *Streptococcus thermophilus* BURD PB 8 (Accession number: MN121741) to enhance both nutritional value and sensory properties. The proximate composition and antioxidant properties of the chocolates were evaluated. The results showed that T_0 had the highest carbohydrate content (42.88±0.01), significantly higher than T_1 (24.62±0.01) T_2 (20.38±0.01), and T_3 (15.65±0.01). Total fat content was substantially higher in T_0 (39.89±0.89) compared to T_1 (4.69±0.30), T_2 (4.42±0.26), and T_3 (3.76±0.33). The antioxidant activity, measured as DPPH radical scavenging activity (%), varied significantly among the treatments. At the beginning, the probiotic viability count of experimental chocolates was 3.80×108 CFU/g. After 60 days of storage, the count dropped to 3.20×108 CFU/g. On day 30, the number of cells had decreased to less than 1000. These findings suggest that the incorporation of *Streptococcus thermophilus* BURD PB 8 with almond milk, oats powder, beetroot powder and stevia powder enhance the nutritional profile and enhances antioxidant activities, making these chocolates a valuable addition to the functional food market.

Keywords: Almond milk, Beetroot powder, chocolate, non-dairy products, probiotics, *Streptococcus thermophilus*, synbiotics

Introduction

Functional foods are gaining popularity due to their potential health benefits beyond basic nutrition (Hasler, 2022) [7]. Synbiotic products, which combine probiotics and prebiotics (Sekhon and Jairath, 2010) [10], are particularly valued for their role in promoting gut health (Maftei, 2019) [8]. Basically, probiotics are live microorganisms that are good for human health. By altering the microflora, secreting antibacterial compounds, competing with pathogens and harmful microorganisms to stop their adhesion, scavenging nutrients required for pathogen survival, creating an antitoxin effect, and partially reversing the effects of infection on the gut epithelium, probiotics have an antimicrobial effect (Tewari *et al*., 2019) [12]. In contrast, prebiotics are indigestible substances that help the colon by promoting the growth and activity of one or a few species of bacteria that are already there (Tewari *et al*., 2023) [13].

This research focuses on the development and analysis (Physico-chemical analysis and probiotic viability count) of non-dairy synbiotic chocolates that have both probiotics and prebiotics, targeting consumers who are lactose intolerant, vegan, or seeking health-enhancing food options.

Materials and Methods

This study was conducted at the department of Food and Nutrition, Swami Vivekananda University, Barrackpore, W.B., India.

In this research Non-dairy Synbiotic Chocolates were prepared according to standard Chocolate-making procedures, with the functional ingredients incorporated during the mixing stage. All raw materials were procured from local commercial suppliers of Behala, Kolkata, W.B., India. The control Chocolate (T_0) was prepared by using cocoa butter, cocoa liquor, whole milk powder, sugar and soy lecithin (Talbot, 2009) [11]. The quantities of different ingredients for the preparation of control Chocolate are tabulated in Table 1

(Talbot, 2009) [11]. In case of experimental Non-dairy Synbiotic Chocolate preparation, oats powder, almond milk, beetroots powder, stevia powder and probiotic microorganisms (*Streptococcus thermophilus* BURD PB 8) were used in different proportion. This new probiotic strain *Streptococcus thermophilus* BURD PB 8 (Accession number: MN121741) was isolated and identified from local curd sample from Purba Bardhaman district of West Bengal, India (Bandyopyadhyay *et al*., 2019) [4].

The newly developed Non-dairy Synbiotic Chocolate formulations were tempered, molded and stored at 4 °C. The quantities of different ingredients for the preparation of experimental Chocolates are tabulated in Table 2.

Table 1: Treatment combination of Control Chocolate (T_0).

Treatments (Control chocolate)	Cacoa powder (%)	Cacoa liquor (%)	Whole milk powder (%)	Sugar Powder (%)	Soy lecithin (gm)
T_0	20	12.4	25.4	43	0.5

In order to prepare the control kulfi (T_0), various percentages of raw components were mixed together to ensure equal blending, including cocoa butter, cocoa liquor, whole milk powder, sugar, and soy lecithin. Following the blending process, the kulfi was moulded after tempering at 45 °C. Cooling was then carried out at between 10 and 15 degrees Celsius (Talbot, 2009) [11].

Table 2: Treatment combination of newly developed Non-dairy Synbiotic Chocolates (T_1, T_2 and T_3)

Treatments (Experimental Chocolate)	Almond milk (mL)	Oats Powder (gm)	Beetroots Powder (gm)	Probiotics (*Streptococcus thermophilus* BURD PB 8) in percentage	Coconut Oil (mL)	Stevia Powder (gm)	Soy lecithin (gm)
T_1	60	30	7	1	2	0.10	0.5
T_2	65	25	5	1	4	0.50	0.5
T_3	70	20	3	1	6	1	0.5

The experimental chocolate formulations detailed in the table vary in their composition of key ingredients to enhance both nutritional value and sensory properties. Treatment T_1 includes 60 mL of almond milk, 30 g of oats powder, 7 g of beetroot powder, 1% probiotics, 2 mL of coconut oil, 0.10 g of stevia powder, and 0.5 g of soy lecithin. Treatment T_2 increases the almond milk to 65 mL and coconut oil to 4 mL, while reducing oats and beetroot powders to 25 g and 5 g respectively, with stevia powder increased to 0.50 g, maintaining the same probiotic and soy lecithin levels. Treatment T_3 further adjusts these proportions with 70 mL of almond milk, 20 g of oats powder, 3 g of beetroot powder, 6 mL of coconut oil, and 1 g of stevia powder, keeping the probiotics and soy lecithin constant. These variations are designed to explore the optimal balance for a healthful, palatable chocolate product.

Analysis of product

Moisture Content (%), Total Ash (%), Protein Content (%), Total Fat (%), Dietary Fibre (%), p^H, Total soluble Solid (Brix) and DPPH (%) were estimated by AOAC (2000) [2] method. And Carbohydrate Content (%) was calculated by difference. To find out the Probiotic Viability count (CFU/g) of the final product**, s**uitable dilutions were poured with respective agar media in sterile Petri dishes in duplicates. In addition to viable count of *Streptococcus thermophilus* BURD PB 8 on MRS agar (after incubation at 37 °C/48 h under facultative anaerobic conditions) (Auty *et al*., 2001) [3].

Results

This study evaluates the proximate composition and antioxidant content of different treatments (T_0, T_1, T_2, and T_3) of a food product. The analyzed parameters include moisture, total ash, carbohydrates, protein, total fat, dietary fibre, p^H, Total soluble Solid and antioxidant percentages. Each parameter was assessed for significant differences across treatments. The values are presented as means ± standard deviations. Significant differences between treatments are indicated by different superscript letters (abcd).

Table 3: Physicochemical analysis of control and experimental non-dairy synbiotic chocolates

Parameters	T_0	T_1	T_2	T_3
Moisture (%)	$2.60{\pm}0.01^{abcd}$	$2.17{\pm}0.01^{bd}$	$2.21{\pm}0.01^{c}$	$2.24{\pm}0.01^{d}$
Total ash (%)	$4.06{\pm}0.01^{abcd}$	$6.47{\pm}0.11^{bcd}$	$5.94{\pm}0.01^{c}$	$4.96{\pm}0.01^{d}$
Carbohydrate (%)	$42.88{\pm}\ 0.01^{abcd}$	$24.62{\pm}\ 0.01^{bd}$	$20.38{\pm}0.01^{cd}$	$15.65{\pm}0.01^{d}$
Protein (%)	$4.06{\pm}0.017^{abc}$	$6.47{\pm}0.01^{bd}$	$5.94{\pm}0.012^{c}$	$4.96{\pm}0.01^{d}$
Total Fat (%)	$39.89{\pm}0.89^{abcd}$	$4.69{\pm}0.30^{b}$	$4.42{\pm}0.26^{c}$	$3.76{\pm}0.33^{d}$
Dietary Fibre (%)	$1.85{\pm}0.02^{abcd}$	$3.26{\pm}0.03^{bcd}$	$2.71{\pm}0.04^{cd}$	$2.18{\pm}0.04^{d}$
p^H	$6.10{\pm}0.05^{acd}$	$6.30{\pm}0.05^{b}$	$6.40{\pm}0.05^{c}$	$6.50{\pm}0.05^{d}$
Total Soluble Solid (Brix)	$44.70{\pm}0.11^{abcd}$	$42.80{\pm}0.05^{bcd}$	$42.40{\pm}0.11^{cd}$	$42.00{\pm}0.11^{d}$
DPPH (%)	$52.0{\pm}0.05^{abcd}$	$72.5{\pm}0.02^{bcd}$	$68.3{\pm}0.05^{cd}$	$64.1{\pm}0.05^{d}$

All the test were performed in triplets. Different letter in the same column indicates statistical significance level of $p<0.0001$.

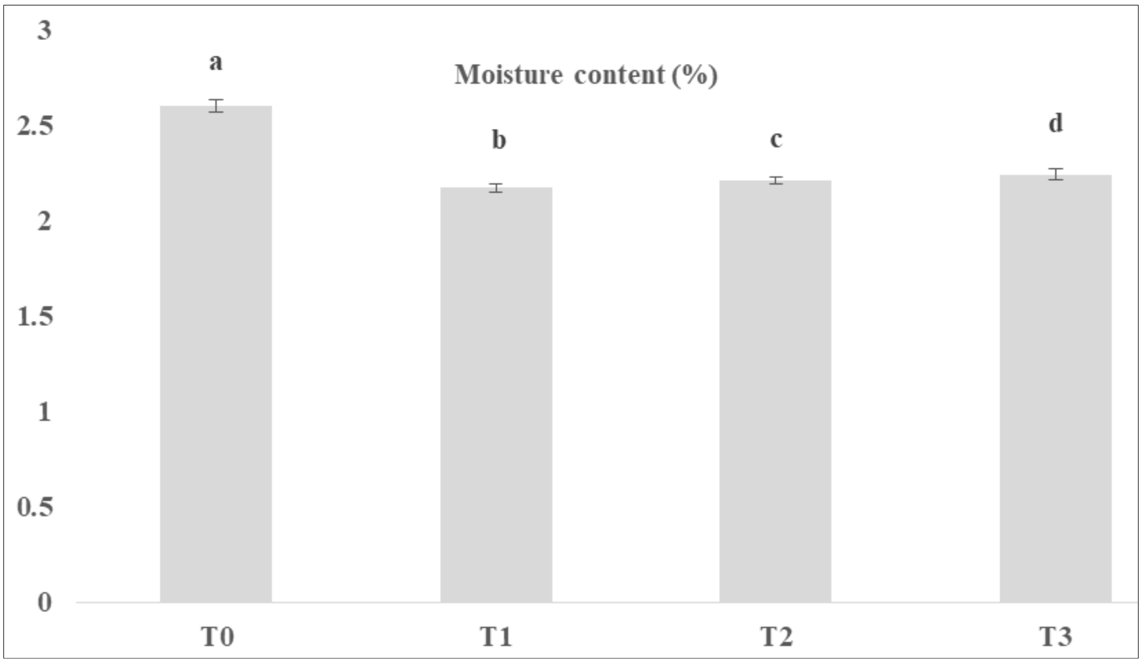

Fig 1: Graphical representation of moisture content (%) of control and experimental non-dairy synbiotic chocolates

The moisture content varied among the treatments, with T_0 (2.600±0.01) having the highest moisture content, followed by T_3 (2.240±0.01), T_2 (2.210±0.01), and T_1 (2.170±0.01). The differences were statistically significant, as indicated by the superscript letters. Lower moisture content in T_1, T_2, and T_3 compared to T_0 suggests that these treatments are more stable and less prone to microbial spoilage, enhancing shelf life.

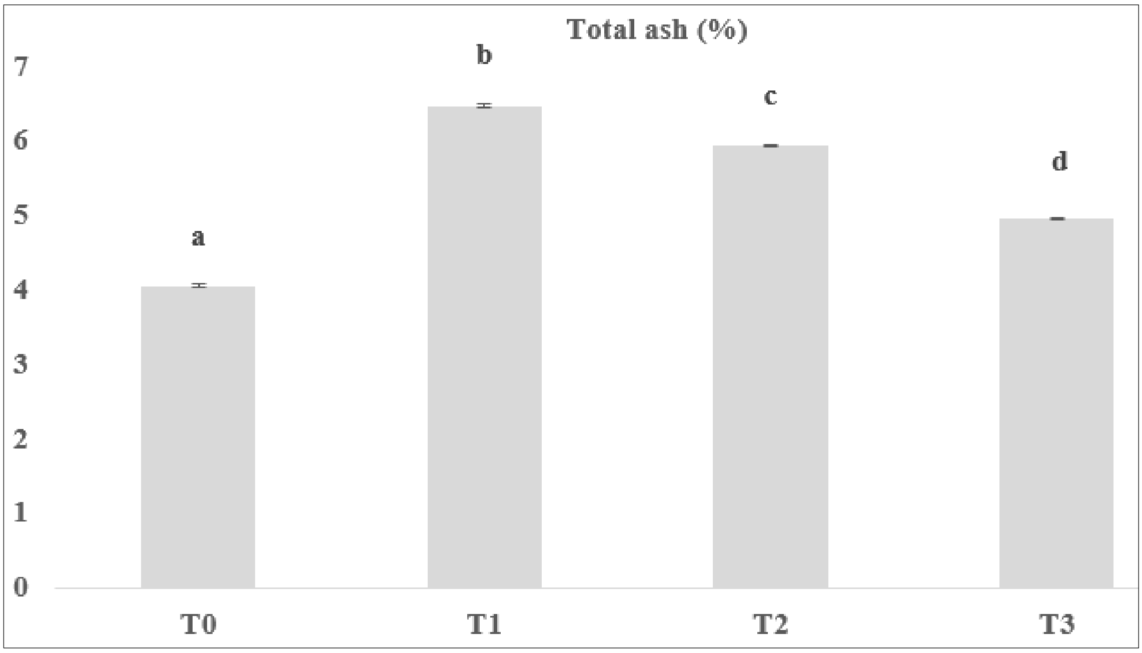

Fig 2: Graphical representation of total ash (%) of control and experimental non-dairy synbiotic chocolates

Total ash content, an indicator of mineral content, was highest in T_1 (6.470±0.11), followed by T_2 (5.940±0.01), T_3 (4.960±0.01), and T_0 (4.060±0.01). The significant increase in ash content from T_0 to T_1 and T_2 suggests that these treatments have a higher mineral content, which could contribute to improved nutritional value. However, the slightly lower ash content in T_3 indicates a decrease in mineral content compared to T_1 and T_2.

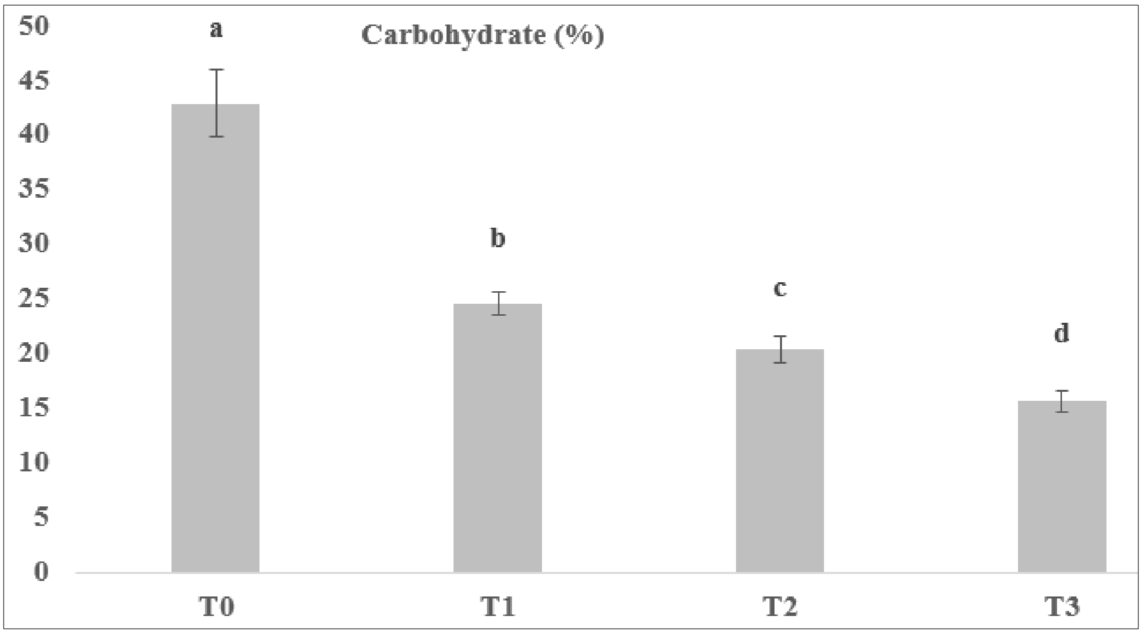

Fig 3: Graphical representation of carbohydrate (%) of control and experimental non-dairy synbiotic chocolates

Carbohydrate content showed a decreasing trend from T_0 to T_3. T_0 had the highest carbohydrate content (42.88±0.017), significantly higher than T_1 (24.62±0.011), T_2 (20.38±0.011), and T_3 (15.65±0.017). This reduction in carbohydrate content across treatments suggests a formulation change that replaces carbohydrates with other components, potentially aiming to create a lower-carb product suitable for specific dietary needs.

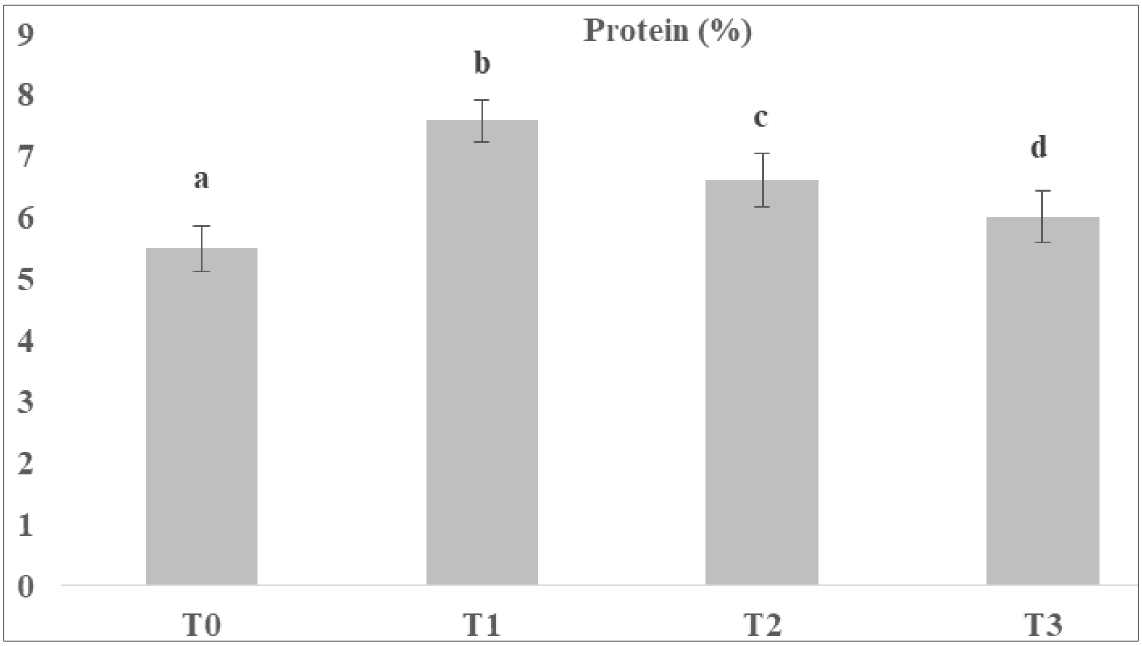

Fig 4: Graphical representation of protein content (%) of control and experimental non-dairy synbiotic chocolates

Protein content was highest in T_1 (6.470±0.011) and T_2 (5.940±0.012), significantly higher than T_0 (4.060±0.017) and T_3 (4.960±0.011). The increased protein content in T_1 and T_2 suggests these treatments were designed to enhance the protein profile of the product, making them more suitable for consumers seeking higher protein intake, such as athletes and bodybuilders.

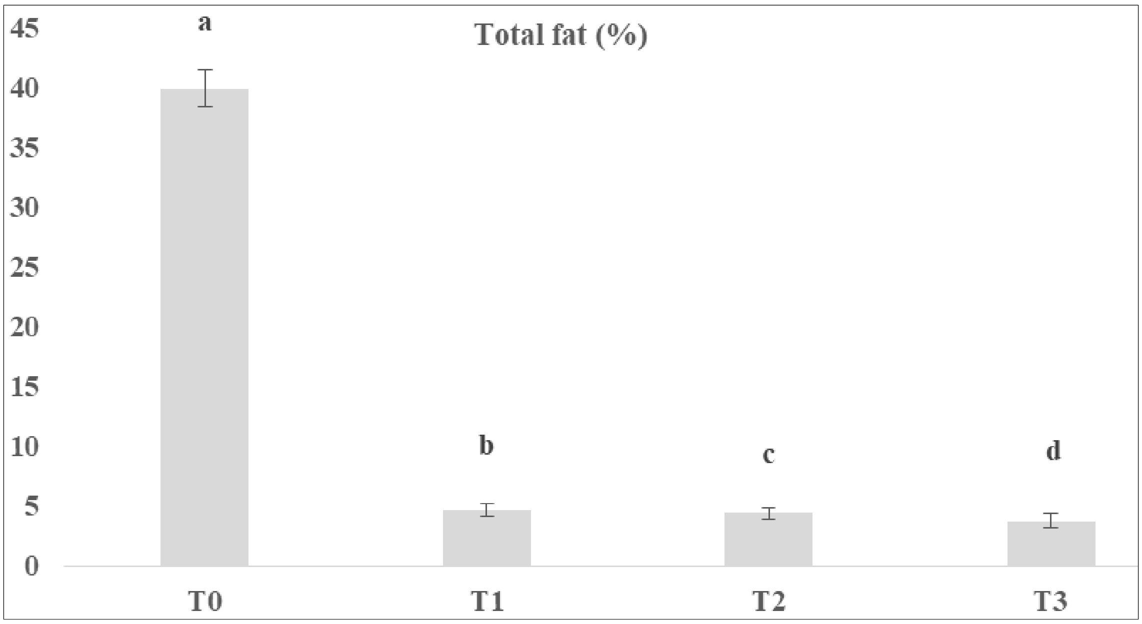

Fig 5: Graphical representation of total fat (%) of control and experimental non-dairy synbiotic chocolates

Total fat content was substantially higher in T_0 (39.89±0.89) compared to T_1 (4.693±0.30), T_2 (4.423±0.26), and T_3 (3.763±0.33). The significant reduction in fat content from T_0 to the other treatments indicates an effort to prepare a lower-fat product. This makes T_1, T_2, and T_3 more suitable for health-conscious consumers aiming to reduce fat intake.

Fig 6: Graphical representation of dietary fibre (%) of control and experimental non-dairy synbiotic chocolates

Dietary fibre content was highest in T_1 (3.260±0.03) followed by T_2 (2.717±0.04) and T_3 (2.183±0.04), with T_0 having the lowest fibre content (1.850±0.02). The increase in dietary fibre in T_1, T_2, and T_3 indicates these formulations are designed to enhance fibre intake, which is beneficial for digestive health and can help in managing weight and blood sugar levels.

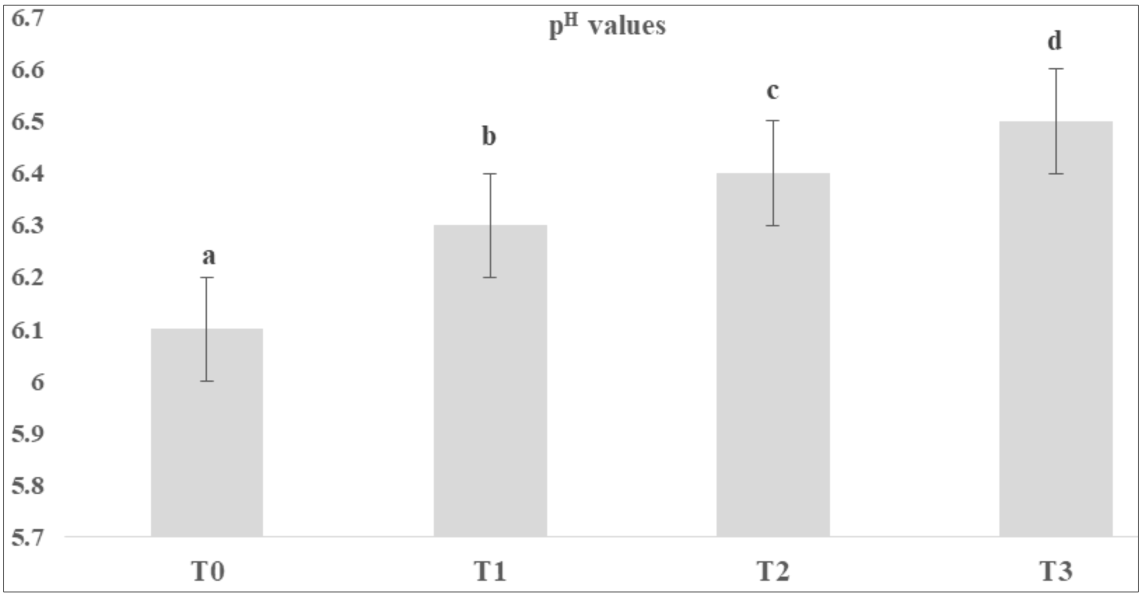

Fig 7: Graphical representation of p^H of control and experimental non-dairy synbiotic chocolates

The p^H values of the Non-Dairy Synbiotic Chocolates varied significantly among the different treatments (T_0, T_1, T_2, T_3). The control sample (T_0) exhibited the lowest p^H of 6.1±0.05, while T3 had the highest p^H of 6.5±0.05. The gradual increase in p^H from T_0 to T_3 suggests that the addition of almond milk, oats powder, and *Streptococcus thermophilus* BURD PB 8 influenced the acidity of the chocolate. Specifically, the fermentation process likely contributed to this p^H increase as the probiotic bacteria metabolized substrates, potentially producing less acidic by products compared to the original mixture.

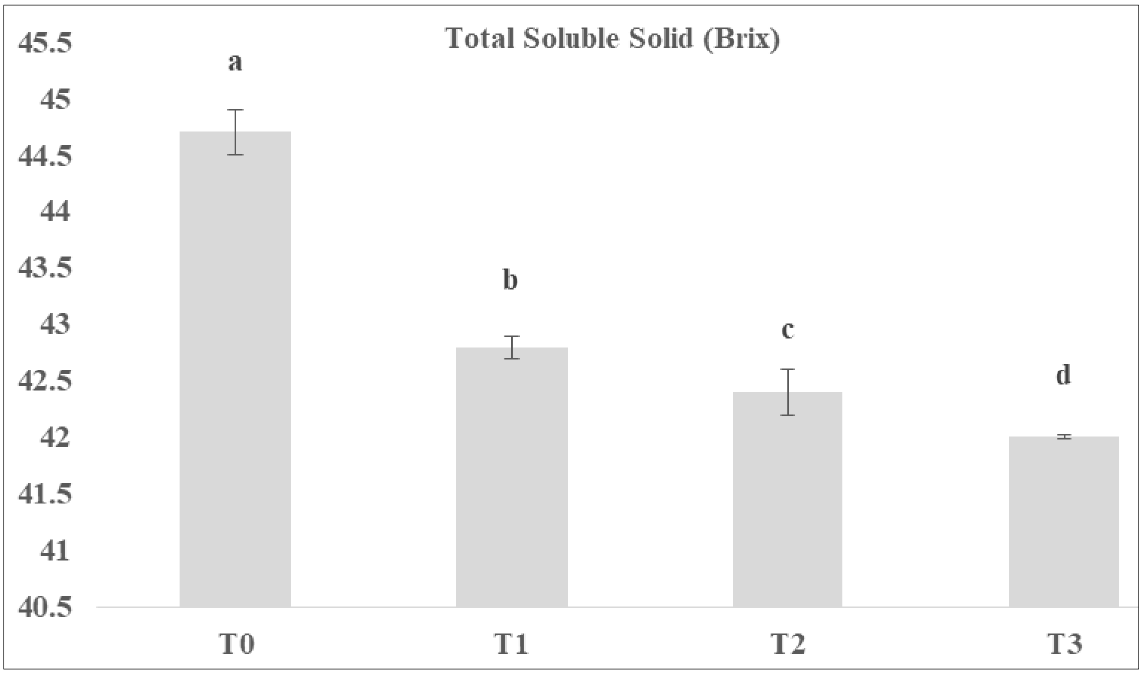

Fig 8: Graphical representation of Total Soluble Solid (Brix) of control and experimental non-dairy synbiotic chocolates

The total soluble solids, measured in degrees Brix (°Brix), also showed significant differences across the treatments. The control sample (T_0) had the highest °Brix value at 44.7 ± 0.11, while the synbiotic chocolate with the highest probiotic content (T_3) had the lowest °Brix value at 42.0±0.11. This decrease in °Brix from T_0 to T_3 indicates that the fermentation by *Streptococcus thermophilus* BURD PB 8 consumed some of the soluble solids, likely sugars, resulting in a lower °Brix value. This aligns with the expected metabolic activity of probiotics, which utilize available sugars for growth and activity.

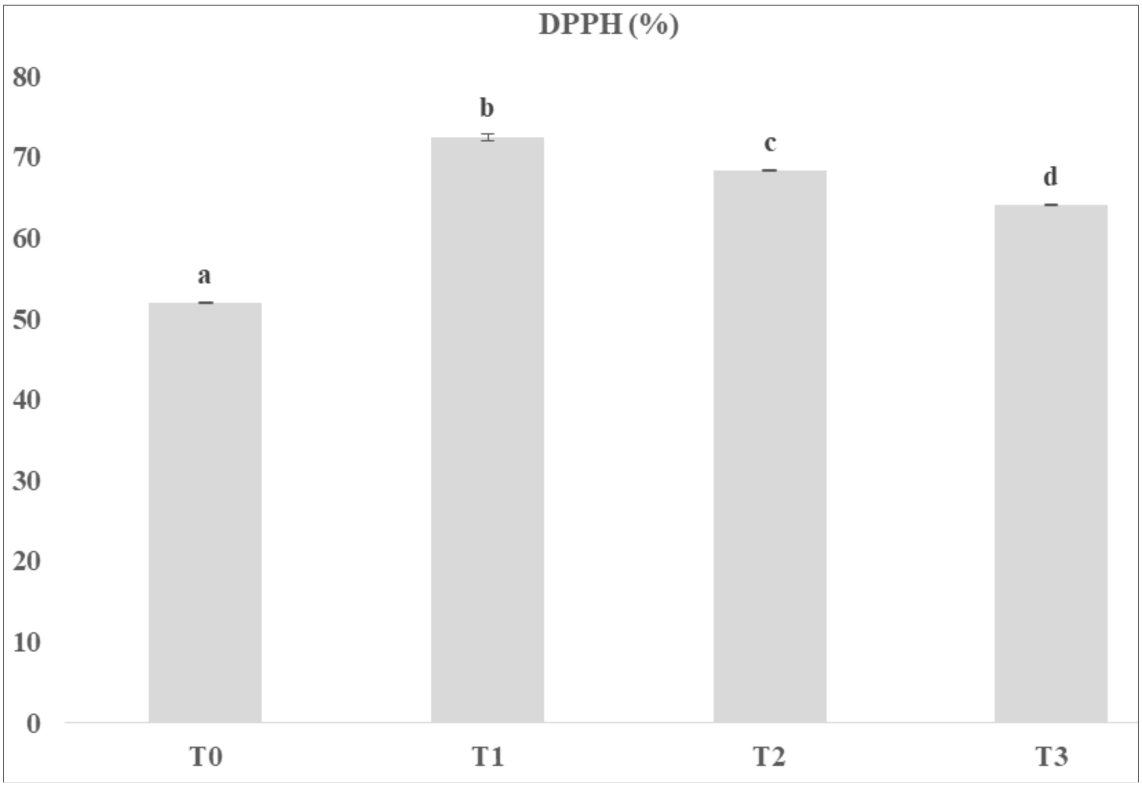

Fig 9: Graphical representation of DPPH (%) of control and experimental non-dairy synbiotic chocolates

The antioxidant activity, measured as DPPH radical scavenging activity (%), varied significantly among the treatments. The control sample (T_0) had the lowest DPPH activity at 52.0±0.05%, whereas T_1 exhibited the highest activity at 72.5±0.02%. The antioxidant activity decreased slightly in T_2 (68.3±0.05%) and further in T_3 (64.1±0.05%). The increase in antioxidant activity from T_0 to T_1 suggests that the initial addition of almond milk, oats powder, and the probiotic improved the antioxidant properties of the chocolate. A similar outcome from a previously published study showed that the use of Galacto-oligosaccharides (GOS) increased the DPPH (%) in chocolate (Tewari *et al*., 2023) [13]. However, the slight decrease in DPPH activity in T_2 and T_3 could indicate that beyond a certain concentration, the components might not synergize as effectively to boost antioxidant properties, or that the fermentation process slightly reduces certain antioxidant compounds.

Probiotic Viability:

The initial CFU count was 3.80×10^8 CFU/g. The count declined to 3.20×10^8 CFU/g during the first 60 days of storage. However, the cell count had fallen below 1000 on the 30th day. To confirm this observation, 1 g of chocolate was suspended in 100 ml sterile skim milk and was incubated at 37 ˚C. After 24 h, the milk was found in coagulated form having 0.8% lactic acid. The cells were not capable to form colonies while plated on MRS. It is difficult to give the exact reason for this, but it could be attributed to the complex internal structure of chocolate. The findings of this study are close to those of Gadhiya *et al*. (2018) [6].

Discussion

The developed non-dairy synbiotic chocolates have advantageous physicochemical characteristics, such as a high protein content that aids in muscle maintenance and repair and a low moisture level that extends shelf life. The fat content supplies important fatty acids and is mostly sourced from almond milk (Vanga and Raghavan, 2018) [15]. The use of oats powder significantly increases dietary fibre content, promoting digestive health (Tosh and Bordenave, 2020) [14].

The observed increase in p^H values with the addition of *Streptococcus thermophilus* BURD PB 8 can be attributed to the metabolic activities of the probiotic, which may produce less acidic byproducts during fermentation. This rise in p^H indicates a shift in the chemical environment of the chocolate, which could affect its flavor and texture (Afoakwa *et al*., 2008) [1].

The decrease in total soluble solids (°Brix) across the treatments demonstrates the effective fermentation by the probiotic, which utilizes soluble components for growth. This is a desirable outcome in synbiotic products as it signifies active fermentation, contributing to the health benefits associated with probiotics (Markowiak and Śliżewska, 2017) [9].

The significant enhancement in DPPH radical scavenging activity in T_1 compared to T_0 highlights the beneficial impact of adding almond milk, oats powder, and probiotics on the antioxidant properties of the chocolate. The slight reduction in antioxidant activity in T_2 and T_3 suggests a potential threshold effect, where optimal concentrations of added components maximize antioxidant properties, beyond which the benefits plateau or decrease.

Overall, the results indicate that the incorporation of almond milk, oats powder, and *Streptococcus thermophilus* BURD PB 8 in non-dairy synbiotic chocolates enhances their functional properties, particularly in terms of p^H stability, reduction in soluble sugars, and improved antioxidant activity, making them a potentially healthier alternative to traditional dairy chocolates.

These findings suggest that the synbiotic chocolates can serve as a nutritious, functional food suitable for various consumer groups, including those with lactose intolerance, vegans, and health-conscious individuals. The incorporation of almond milk, oats powder, and *Streptococcus thermophilus* BURD PB 8 not only improves the nutritional profile but also offers additional health benefits, making these chocolates a valuable addition to the functional food market.

Conclusion
The physicochemical analysis of the newly developed non-dairy synbiotic chocolates enriched with almond milk, oats powder, and *Streptococcus thermophilus* BURD PB 8 demonstrates their potential as a functional food product. The enhanced nutritional profile and probiotic benefits make these chocolates suitable for health-conscious consumers and those with specific dietary requirements.

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Conflicts of interest: The authors declare no conflict of interest.

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