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RESEARCH ARTICLE

Nutritional and Functional Properties of Prebiotic Enriched Chocolates

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Abstract

Objectives: To demonstrate the nutritional and functional characteristics of the developed phytochemical containing prebiotic-enriched chocolates. **Methods:** Methods of Association of Official Analytical Chemists and standard biochemical procedures were employed for the determination of nutrient and phytochemical composition. Antioxidant and prebiotic activity were calculated through DPPH, the reducing potential assay, as well as the growth kinetics and plate count of *Lactobacillus rhamnosus* GG, respectively ($p \leq 0.01$, 95% confidence interval). The functionality of the products was also analyzed in an *in vitro* simulated human intestinal digestion environment. **Findings:** Variation 3 (V3) chocolates prepared through the incorporation of cocoa powder (64%), cocoa butter (22.5%), stevia (3%), inulin (10%), and soy lecithin (0.5%), scored the highest sensory rating amongst all the prepared products. V3 revealed a desired proportion of proximate components (low fat and high fibre), minerals, vitamins, and phytochemicals such as polyphenol (25mg/g), alkaloids (189 mg/mL), and flavonoids (46mg/g), versus the standard chocolate (S), along with a low calorific value of the former (541.2Kcal). Furthermore, V3 could efficiently scavenge 2, 2-diphenyl-1-picryl-hydrazyl (86.04±0.4%), display reducing power (2.84±0.02), and accelerate the growth of probiotic *Lactobacillus rhamnosus* GG (OD0.59, 11.61 Log CFU/mL at 20 hours), portraying antioxidant and prebiotic properties. Interestingly, V3 was found to sustain a significant proportion of antioxidant and prebiotic potential under imitated intestinal digestion, indicating their effectiveness in propagating benefits in the human body post-consumption. **Novelty**: Considerable retention of the antioxidant and prebiotic effects post intestinal digestion, besides the nutritional and phytochemical constitution of V3, may promote well-being consequent to its adaption alternative to conventional chocolates.

Keywords: Antioxidant; Chocolate; Nutrients; Phytochemicals; Prebiotic

1 Introduction

Chocolates are one of the highly preferred food products worldwide owing to their attractive organoleptic characteristics. The chemosensory qualities of chocolate, including sweetness and an alluring aroma, cause consumers to develop a natural craving for them [1]. Interestingly, the demand for this commodity is anticipated to expand to 161.1 billion United States dollars in the year 2026 at a compound annual growth rate of 3% [2]. Contemplating the demand for chocolate and its recognition as a suitable carrier for functional ingredients has led to increased efforts towards improving the components' general functionality as well as amplifying the quality and nutritional content for strengthening the overall health status of consumers [3,4]. Therefore, the reformulation of chocolates to create a product that is high in polyphenolic phytonutrients and prebiotic fibres as well as low in calories and sugar may improve the advantages associated with their consumption. Inulin, a potent prebiotic, has been shown to stimulate the growth of beneficial bacteria such as species of *Lactobacillus, Bifidobacterium*, and *Faecalibacterium* in the intestine, leading to protection against diseases as well as fostering nutrient absorption [5]. Moreover, polyphenolic compounds, especially flavanols in cocoa powder, have also been associated with benefits such as blood flow synchronization, reduction of hypertension, and anti-inflammatory potential. Cocoa butter is a predominant source of saturated and unsaturated fatty acids such as stearic, palmitic, and linoleic acids that may be employed to produce chocolates, which, owing to its unique structure, results in lower incidences of fat-mediated deleterious effects compared to other fat-containing ingredients [6]. Additionally, stevia, a safe, zero-calorie, and low-glycemic natural sweetener, and soy lecithin, an emulsifier, have been known to reduce caloric intake and improve the viscosity of the product, thereby exacerbating the positive effects while maintaining the desired rheological characteristics [7]. Noteworthy, loss of nutrients and functional effectiveness have been reported to occur during the process of human digestion, limiting the bioavailability of the important compounds in the food product [8]. Moreover, previous reports on prebiotic and synbiotic chocolates have pointed out the requirement of conducting bioavailability studies to understand the actual efficiency of the products inside a human host [9]. Although attempts have been made in the recent past to develop confectionary items with health-promoting properties, limited research has been carried out to evaluate their effectiveness post-human gastrointestinal digestion [10]. Therefore, the present study aimed to formulate a prebiotic-enriched, phytochemical-containing low-calorie chocolate containing inulin, cocoa powder, cocoa butter, soy lecithin, and stevia, to deliver a health-promoting product without compromising the taste and sensory attributes that consumers expect from this confection and analyze its nutritional and functional attributes. Importantly, the study specifically focuses on assessing the retention of the functional properties of the developed chocolates post a simulated human intestinal digestion environment to not only estimate their potency in the human body but also adequately contribute towards propagating the advantageous effects mediated through their consumption as an alternative to conventional chocolates.

2 Methodology

2.1 Product Development and Sensory Evaluation

The standard recipe (S) for chocolate was prepared by the amalgamation of cocoa powder, cocoa butter, sugar, and soy lecithin. Additionally, prebiotic inulin and sweetener stevia were added to the variations owing to their local availability and their nutritional and functional potentials. Four different variations (V1 to V4) of the product were prepared according to the proportions mentioned in Table 1. The group of 50 randomly selected panellists (18-24 years old) assessed the sensory qualities of the value-added items. The U.S. Army Quartermaster Food and Container Institute's 9-point hedonic scale was used to evaluate the organoleptic quality of the developed products [11].

Table 1. Formulationsof Prebiotic Chocolates

S.No.	Raw Material ↓ / Formula →	Standard Recipe (S)	Variation 1 (V1)	Variation 2 (V2)	Variation 3 (V3)	Variation 4 (V4)
1	Cocoa powder (%)	55	68	66	64	62
2	Cocoa butter (%)	20	23	23	22.5	22.5
3	Icing sugar (%)	24.5	-	-	-	-
4	Stevia (%)	-	3.5	3.5	3	3
5	Inulin (%)	-	5	7.5	10	12
6	Soy lecithin (%)	0.5	0.5	0.5	0.5	0.5
	Total (%)	100	100	100	100	100

2.2 Analysis of Proximate Components and Nutrients

The prebiotic chocolate variation that scored the highest sensory rating was assayed for proximate components and important nutrients compared to the standard recipe. The hot air oven drying method and hydrometric measurement of the samples in the furnace at 600°C were used to monitor the moisture and ash content, respectively. The Biuret and the Soxhlet methods were employed to estimate the products' protein and fat proportions. The Anthrone assay was utilized to assess the total carbohydrate concentration of the chocolates [12]. The percentage of crude fibre was determined by using the acid-base digestion method with 1.25% H_2SO_4 and 1.25% NaOH solutions. The content of saturated, and trans fat was determined through ICMR-based calculation [13]. B vitamin analysis (B12 and B6) was conducted by UV-visible spectrophotometer at 363nm and 290nm, respectively. Phosphorus, calcium, iron, and sodium content were measured by the Molybdate U.V. method using the Phosphorus kit (Tulip Diagnostic, India), o-Cresolphthalein Complexone (OCPC) method via the Calcium kit (Coral Clinical System, India), Ferrozine method by utilizing the Iron kit (Tulip, India), and β-galactosidase based colorimetric assay (Coral Clinical System, India). Zinc was measured by EDTA-dependent complexometric titration. Vitamins A, E, and D were evaluated by techniques reported by Adegbaju et al. and Rahman et al. [14,15].

2.3 Determination of Phytochemicals

The chocolates were assayed for phytochemicals such as total phenols, flavonoids, and alkaloids. The total phenols were estimated with Folin Ciocalteau's phenol reagent. The absorbance at 765 nm was calculated using a UV-VIS spectrophotometer. The aluminium chloride ($AlCl_3$) method was employed to calculate the total flavonoid content at 510 nm absorbance against quercetin as a standard. A titrimetric method was utilized to calculate the total amount of alkaloids. A 100 mL separating funnel was filled with 10 mL of the sample extract and 10 mL of 0.1N HCl, allowed to stand for two to three minutes, and forcefully shaken, leading to the solubilization of alkaloids. The beaker's solutions were titrated against 0.1N NaOH until the red colour turned pale yellow. The total alkaloid content was estimated by considering 1mL (0.1N) NaOH to be equivalent to 0.0162 g alkaloid.

2.4 Determinations of Antioxidants

The antioxidant activity of the products was calculated using the DPPH radical scavenging assay and the reducing power estimation method. 0.1 mM DPPH solution was prepared, and 2.4 mL of this solution was combined with 1.6 mL of extract at various doses (20-100 μg/mL), vortexed, placed at room temperature in the dark for 30 minutes, and absorbance was measured at 517 nm (UV-visible spectrophotometer, Hitachi; U-2910 Spectrophotometer, Japan) versus standard ascorbic acid. The reduction property of the extracts was assessed according to the method of Bhalodia [16]. Different concentrations (0.2-1.0 mg/mL) of extracts were added to 1 mL of distilled water, 2.5 mL of 0.2 M phosphate buffer (pH 6.6), and 2.5 mL of 1% potassium ferrocyanide, incubated (50°C for 20 min) and precipitated with trichloroacetic acid. The resulting mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was diluted with distilled water (1:1) and 0.1% $FeCl_3$, followed by absorbance estimation at 700 nm.

2.5 Prebiotic Potential

De Man Rogosa Sharpe (MRS) broth was used to assess the *in vitro* prebiotic potential of the chocolates. MRS medium containing 2% chocolate extract and 1% sub-cultured broth (*Lactobacillus rhamnosus* GG, ATCC 53013) at 37°C was incubated in a nephelometer under anaerobic conditions. The optical density (OD) at 700 nm was measured every hour for 24 hours to determine the growth [17]. The prebiotic potential was also estimated by the standard plate count (SPC) method. Serial dilutions of the sample were pour-plated with 1% *L. rhamnosus* on pre-sterilized MRS agar (121°C and 15psi) and the growth of *Lactobacillus* was monitored post 20 hours of incubation at 37 °C. Experiments were repeated in triplicate, and the results were represented as Log CFU/mL. MRS media with *L. rhamnosus* without any chocolate extract was used as the control.

2.6 *In vitro* Intestinal Digestion

A simulated intestinal digestive solution was prepared with 125.0 mM NaCl, 0.6 mM $CaCl_2$, 0.3 mM $MgCl_2$, trypsin (11 U/mL), α-chymotrypsin (24 U/mL) and pancreatic lipase (590U/mL). 5 grams of standard or developed products were subjected to treatment with the above solution for 60 minutes and thereafter assayed for their functional characteristics.

2.7 Cost Evaluation

The cost of the product was estimated by using the following formula: Price of the product + 15% profit + 15% processing cost+ 20% labor cost + 20% overhead cost = Total price

2.8 Shelf-life Studies

Nutrient agar was prepared for the determination of shelf life. The shelf life was evaluated by measuring the microbial growth at regular time intervals upon storage at room temperature (25°C) as well as at refrigeration temperature (4°C). 0.1mL of serially diluted aliquots of the sample were spread plated on nutrient agar and incubated at 37°C for 24-48 hours for the determination of growth. Results were depicted as colony-forming units (CFU)/mL. The growth of the colony was calculated by the following formula: CFU/mL = (Number of colonies × dilution factor)/Volume of culture plate.

2.9 Statistical Analysis of the Data

Results was analysed by using the data analysis pack of Microsoft Office Excel (2010, India). They were represented as mean±sem of triplicate experiments. P value was calculated by using the T TEST feature of Microsoft Excel 2010 to determine the significance of the data obtained. Only p value, significance expressed as $p \leq 0.0001$=***, $p \leq 0.005$= ** and $p \leq 0.01$= * was considered as statistically significant with CI: 95% confidence interval.

3 Results and Discussion

3.1 Sensory Acceptance of the Developed Chocolates

The developed products were evaluated for sensory properties, owing to their importance in determining consumer acceptance and quality of the product. Results revealed that variation 3 (V3) was ranked the highest in the overall sensory characteristics with an average ranking of 8.7 versus S, denoting its extreme acceptance by the panel members owing to its clear appearance, smooth texture, good taste, and sweet odour (Table 2). Variation 2 (V2) was ranked 7.8, which stated that it was moderately appreciated by the respondents. Standard recipe and variation 1 (V1) received an average ranking of 7.0 and 7.4, indicating their modest acceptance by the panel. Variation 4 (V4) obtained the lowest ranking of 5.3 versus the other products as per the 9-point hedonic scale, implying that the taste, appearance, and texture of variation 4 were least accepted, which may be because of the increased addition of inulin. Hence, V3 was the most accepted chocolate based on its organoleptic properties. The results of this study align with previous research indicating that the combination of cocoa powder with inulin is an effective approach for enhancing consumer acceptance of a product [18]. Specifically, this study demonstrated that the sensory attributes of chocolate including dark brownish colour, shape, odour, taste, and mouthfeel were the most significant factors determining their acceptance. The combination of cocoa powder with inulin represents a promising approach to improving the sensory characteristics of developed products, as demonstrated by the results of this study [19].

Table 2. Sensory Evaluation of the Products

Parameters	Standard (S)	Variation 1 (V1)	Variation 2 (V2)	Variation 3 (V3)	Variation 4 (V4)
Appearance	8.03±0.19	8.26±0.17**	8.26±0.16**	8.3±0.21**	6.6±0.12*
Colour	8.4±0.12	8.3±0.17**	8.3±0.21**	8.5±0.14**	8.06±0.18**
Taste	7.1±0.23	7.5±0.18**	7.5±0.21*	8.8±0.20**	4.6±0.19*
Texture	7.6±0.21	7.4±0.23**	7.8±0.20*	8.1±0.19**	4.6±0.22*
Odour	7.8±0.13	7.8±0.12**	8.1±0.12**	8.2±0.16**	6.9±0.14*
Overall ranking	7.0±0.18	7.4±0.16**	7.8±0.12**	8.7±0.18**	5.3±0.12*

Note: ***, **, * denotes $p \leq 0.0001$, $p \leq 0.005$ and $p \leq 0.01$, respectively at 95% CI

3.2 Nutrient and Phytochemical Analysis

Since variation 3 chocolates qualified as the most accepted product through sensory evaluation, these were further evaluated to determine their nutrient, and phytochemical composition versus the standard product to completely demonstrate the quality and health benefits that may be obtained through their intake. Results displayed that V3 harboured low moisture, probably due to the moisture absorption ability of inulin and high ash content versus the standard chocolate, indicating a greater mineral

content in the former. Moreover, V3 was found to contain 46.5 g/100 g, 7.8 g/100 g, and 36 g/100 g of carbohydrate, protein, and fat, respectively, compared to 40.9 g/100 g, 5 g/100 g, and 45 g/100 g in S (Table 3). The addition of inulin and the high amount of cocoa powder in the variation (V3) may be responsible for the increased carbohydrate percentage in this product, as also discussed in previous studies [20]. Moreover, the elevated addition of cocoa powder may be ascribed to the heightened protein percentage in this product (V3), since no other protein-containing ingredient was used in the same. Notably, the lowered fat content, including low saturated and trans fat, in variation 3 versus standard chocolate may be due to the utilization of an increased proportion of inulin, which acts as a bulking agent and a fat replacer, as well as the reduced addition of cocoa butter, making them suitable for obese patients. Interestingly, the sugar content of the products was restricted through the utilization of stevia, thereby proving helpful for individuals following a sugar-restricted diet. The nutritional evaluation also revealed the presence of 22.4% crude fibre in the best variation of the developed chocolate (V3), mainly due to the inclusion of optimum amounts of inulin in this product. Micronutrient estimation displayed the existence of 3.76 mg/dL, 4.3 mg/dL, 329.69 μg/dL, 1.65 mg/100g, and 10.52 mg/100g phosphorus, calcium, iron, zinc, and sodium in V3 chocolates, respectively, which may be attributed to the proportion of ingredients, especially the increased cocoa powder addition in these products. Previous findings have also revealed that the inclusion of cocoa powder significantly benefits the micronutrient profile of the sample [21]. Furthermore, Variation 3 chocolates displayed a higher content of important phytonutrients compared to the standard product. Indeed, V3 chocolates were found to contain 25 mg/g polyphenols versus only 15 mg/g in standard chocolate (Table 3) which may be due to increased utilization of cocoa powder that has previously been reported to harbor total phenols inclusive of polyphenols [22]. Moreover, V3 also manifested 189 mg/ml of alkaloids and 46 mg/g flavonoids due to its cocoa powder content. V3 also displayed the notable presence of fat-soluble vitamins (A, D, and E) along with vitamins B6 and B12 (Table 3), qualifying as a good source of micronutrients that may help satisfy a substantial proportion of the recommended daily intake (RDI) per serving in contrast to numerous earlier articles that have mainly assessed the macronutrient profile with little emphasis on the micronutrient and phytochemical composition [6,23]. Therefore, consumption of these chocolates may benefit overall health not only due to their nutrient and phytonutrient content but also because of them being a low-fat and low-sugar alternative.

Table 3. Nutrient and Phytochemical Content

Component	Standard (S)	Variation 3 (V3)	Component	Standard (S)	Variation 3 (V3)
Moisture content (%)	3.0± 0.9	5.0± 0.2**	Phosphorus (mg/dL)	3.21±0.8	3.76 ±1.0*
Ash content (%)	82.0± 0.8	90.0± 0.1***	Calcium(mg/dL)	3.7±1.4	4.3 ±1.2*
Energy (Kcal)	588.6±0.34	541.2±0.28**	Iron(μg/dL)	288.31±0.06	329.69±0.8***
Carbohydrate (g/100g)	40.9±0.02	46.5±0.02**	Vitamin A (IU)	110.0±0.5	112.80±0.5
Protein (g/100g)	5.0±0.3	7.8±0.2*	Vitamin D (IU)	0.09±0.003	0.12±0.001*
Fat (g/100g)	45.0±0.04	36.0±0.06**	Vitamin E (IU)	0.09±0.004	4.06±0.02**
Crude fibre (%)	16.1±0.03	22.4±0.02**	Vitamin B6 (mg/100g)	0.125±0.04	0.155±0.03*
Saturated fat (g/100g)	2.65	2.54	Vitamin B12 (μg/100g)	0.12±0.005	0.15±0.002*
Trans fat (g/100g)	0.40	0.30	Polyphenol (mg/g)	15.0±0.2	25.0±0.1**
Zinc (mg/100g)	1.53±0.05	1.65±0.04	Alkaloids (mg/mL)	153.0±0.03	189.0±0.01***
Sodium (mg/100g)	9.02±0.11	10.52±0.13	Flavonoids (mg/g)	29.5±1.0	46.0±0.8***

Note: ***, **, * denotes $p \leq 0.0001$, $p \leq 0.005$ and $p \leq 0.01$, respectively at 95% CI

3.3 Antioxidant Potential Before and After *In Vitro* Intestinal Digestion

Since the food products were made with cocoa, which is known for antioxidant action, and because the chocolates were observed to contain a significant proportion of polyphenols, flavonoids, and alkaloids, these products were assayed for their antioxidant potential via estimation of their abilities to scavenge DPPH free radicals and portrayal of reducing power. As noted in Table 4, variation 3 chocolates manifested a higher DPPH radical foraging property (86.04±0.4%) than the standard product (72.02±0.6 %). Numerous studies have exhibited the high antioxidant properties of cocoa-rich chocolates by demonstrating increased DPPH radical foraging ability. This study additionally estimated the reducing power of the chocolates to further analyze their antioxidant capacity. V3 manifested a higher reducing potential in contrast to S, as tabulated in Table 4. In contrast to earlier reports, the present study additionally evaluated the retention of antioxidant properties after simulated *in*

vitro intestinal digestion to estimate the effectiveness of the product inside the human body. Interestingly, although both samples displayed a reduction in antioxidant potential after treatment in an intestinal environment, V3 was observed to retain the former significantly (81.21±0.8% DPPH inhibition and 2.08±0.01 μg/mL reducing power) versus S (61.12±1.01% DPPH inhibition and 0.59±0.001 μg/mL reducing power) (Table 4). As also noted in previous studies, cocoa has high concentrations of phenolic antioxidants, alkaloid compounds, and flavonoids like catechin, epicatechin, and procyanidins, which may be responsible for the observed antioxidant capacity of the developed products and may be supportive in the prevention of cellular damage by terminating free radical formation [24]. Hence, consumption of V3 chocolates may benefit health through their antioxidant potential, mainly attributed to their cocoa content, especially due to their considerable capacity to retain these properties post-*in vitro* intestinal digestion.

Table 4. Antioxidant Potential Before and After *In Vitro* Intestinal Digestion

Product	DPPH Assay (% inhibition)	Reducing Power Assay (μg/mL)
Standard (S)	72.02±0.6	0.73±0.001**
Standard (S) after intestinal digestion	61.12±1.01**	0.59±0.001**
Variation 3 (V3)	86.04±0.4***	2.84±0.02**
Variation 3 (V3) after intestinal digestion	81.21±0.8**	2.08±0.01**

Note: ***, **, * denotes $p \leq 0.0001$, $p \leq 0.005$ and $p \leq 0.01$, respectively at 95% CI

3.4 Prebiotic Properties of the Developed Chocolates Before and After *In Vitro* Intestinal Digestion

The developed chocolates were tested for their prebiotic potential due to the presence of considerable quantities of polysaccharides, including fibre, as well as phytochemicals that have previously been linked with prebiotic properties. In comparison to the control (without chocolate extract), both standard and V3 chocolates increased the growth of *Lactobacillus rhamnosus* GG with increasing time. Accelerated growth was noticed with fibre-rich (Inulin) chocolate, as manifested by optical density values of 0.59 (V3) and 0.44 (S) compared to 0.09 in the control sample at 20 hours (Figure 1**A**). All samples were observed to promote the highest growth of *L. rhamnosus* at 20 hours, after which they were found to gradually enter the stationary phase followed by the death phase (Figure 1 **A**). Additionally, V3 chocolates also manifested increased prebiotic potential displayed by a higher colony count (11.61 Log CFU/mL) versus the standard (8.83 11.61 Log CFU/mL) at 20 hours (Figure 1 **B**). The human gastrointestinal environment may affect the net functionality of the products. However, limited research so far has evaluated the effect of human intestinal digestion on the prebiotic potential of chocolates. Interestingly, the present result demonstrated sufficient retention of prebiotic potential in V3 post *in vitro* intestinal digestion as manifested by O.D. values of 0.50 and colony count of 9.74 Log CFU/mL versus S (O.D. values of 0.38 and colony count of 6.51 Log CFU/mL) at 20 hours (Figure 1). Notably, although all products showed prebiotic potential, variation 3 (the best variation of the sample) displayed the highest activity even after being subjected to an *in vitro* simulated intestinal digestive environment. Scientific reports have correlated prebiotics with therapeutic benefits in addition to improving overall health [25]. Noteworthy, the considerable prebiotic potential demonstrated in the developed chocolates (V3) may be because of the combination of optimum amounts of cocoa powder and inulin that may be contributing to the desired amounts of soluble fibre, polyphenols, flavonoids, and alkaloids in comparison to other prebiotic chocolates prepared in earlier studies [26]. Furthermore, adequate retention of the prebiotic potential by V3 after *in vitro* intestinal digestion indicates the usefulness of these chocolates towards the maintenance of ideal gut homeostasis.

3.5 Cost Evaluation of the Developed Products

The most approved variation (V3) was calculated to cost Indian rupees (₹) 140.78 per 100 grams, versus ₹105.68 per 100 grams for the standard chocolate (Table 5). The higher content of inulin and stevia utilization in the former may be responsible for the observed cost. In contrast to several previous studies conducted on the development of prebiotic chocolate, the present work estimated the cost due to the importance of this parameter in ensuring that the product is profitable and sustainable in the long run. The price of developed products was found to be comparable to most of the commercially available chocolates, thereby portraying their affordability across people belonging to a range of economic backgrounds. Therefore, the developed product can not only be easily prepared and purchased but may also contribute to additional health benefits compared to regular chocolates owing to their nutritional and functional contents.

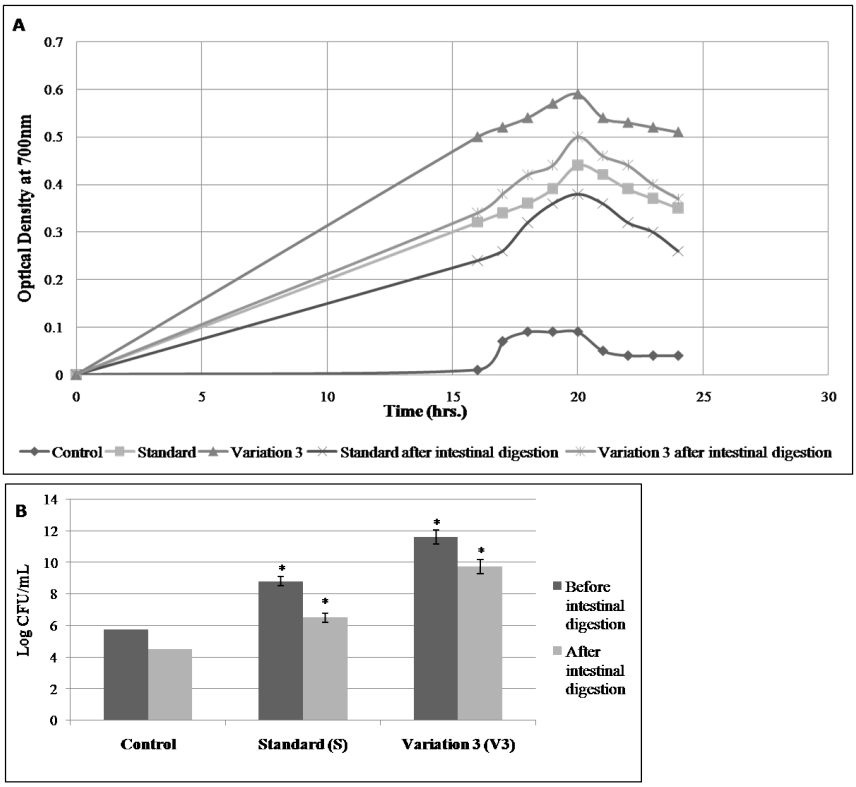

Fig 1. Prebiotic Properties of the Developed Chocolates Before and After *In Vitro* Intestinal Digestion. [A] Growth Kinetics of *Lactobacillus rhamnosus* GG [B] Colony Count of *Lactobacillus rhamnosus* GG (Log CFU/mL))

Table 5. Cost Estimation of the Developed Products

S.No.	Raw Material	Rate (₹)	Quantity Require (g)		Cost (₹)/100g	
			Standard (100g)	Variation3 (100g)	Standard	Variation 3
1	Cocoa powder	100/100g	55	64	55	64
2	Cocoa butter	129/100g	20	22.5	25.8	29.02
3	Icing Sugar	20/100g	25	-	5	-
4	Stevia	110/100g	-	3	-	3.3
5	Inulin	200/100g	-	10	-	20
6	Soy lecithin	350/100g	0.5	0.5	1.75	1.75
					87.55	118.07
15% Profit					100.68	135.78
Miscellaneous cost					5	5
Total cost					105.68	140.78

3.6 Shelf Life of the Developed Products

Shelf life denotes the time recommendation for storage of food products to retain their defined quality and acceptability, including sensory, chemical, physical, and biological properties, under specified conditions. As observed in Table 6, no notable microbial growth occurred within 20 days of storage at both room temperature (25°C) and refrigeration temperature (4°C). Limited microbial colonies were visible from day 20, and significant growth was visible after 28 days of storage at both temperatures. As noted in several studies, microbiological growth in food culminates in food spoilage with the development of undesirable sensory characteristics, thus making the product unsafe for consumption [27]. Moderately low growth was observed in cases of refrigerated storage compared to chocolates stored at room temperature (Table 6). Interestingly, prior reports on dark chocolates have also documented significant microbial growth after 21 days of storage at 20°C and 30°C [28]. Nonetheless, few reports are available on the assessment of storage at refrigerated conditions (4°C). The present study displays a difference in the intensity of microbial growth at the different temperature conditions (25°C and 4°C), thereby providing valuable information for consumers and manufacturers to make informed decisions about the storage and shelf life of the product. This may also lead to the development of new storage recommendations or packaging solutions to extend the shelf life of the product. Results showed that the developed chocolates could be safely consumed before 20 days post-preparation since no preservatives were used in their composition. Nonetheless, the shelf life may be extended through the use of appropriate preservatives in the recommended dosages.

Table 6. Shelf Life of the Developed Products

Time (day)	Standard (CFU/mL)		Variation 3 (CFU/mL)	
	Room Temperature Storage (25°C)	Refrigerated Storage (4°C)	Room Temperature Storage (25°C)	Refrigerated Storage (4°C)
0	0	0	0	0
4	0	0	0	0
8	0	0	0	0
12	0	0	0	0
16	2	1	2	1
20	8	6	7	5
24	15±2	10±2	12±3	10±3
28	200±10	80±5	185±12*	60±4*

Note: * denotes $p \leq 0.01$ at 95% CI

4 Conclusion

Chocolates are popular worldwide because of their alluring sensory qualities. Nonetheless, they may negatively impact health due to their high sugar and fat content, despite several potential benefits. Therefore, the goal of this study was to formulate a low-fat, low-sugar, phytochemical and prebiotic-rich chocolate. The standard chocolate was developed along with several variations (variations 1, 2, 3, and 4) by the amalgamation of calculated amounts of ingredients like cocoa powder, cocoa butter, stevia, inulin, and soy lecithin. Variation 3 was found to score highest in the sensory parameters amongst all products via the 9-point hedonic scale (8.7±0.18), mainly due to the inclusion of optimum proportions of inulin and cocoa. V3 was found to contain desired amounts of proximate components demonstrated by low fat (36±0.06 g/100g), high fibre (22.4±0.02%) and significant micronutrients including iron (329.69 μg/dL), calcium (4.3 mg/dL), phosphorus (3.76 mg/dL), zinc (1.65 mg/100g), and sodium (10.52 mg/100g) as well as vitamins (A, D, E, B6, and B12), mainly fostered by the utilization of cocoa, inulin, and stevia, thereby indicating their health benefiting potential promoted by their nutrient profiles and by them being a low-fat and low-sugar alternative. Moreover, they were also observed to be good sources of health-promoting phytochemicals such as polyphenols (25±0.1 mg/g), flavonoids (46±0.8 mg/g), and alkaloids (189±0.01mg/mL) through the inclusion of cocoa powder. The addition of inulin fibre along with cocoa phytonutrients bestowed prebiotic properties on the product, as portrayed by the ability of V3 chocolates to promote the growth of *Lactobacillus rhamnosus* GG, implying their gut-beneficial properties. Additionally, inulin was also employed to restrict the total caloric content of the product by acting as a bulking agent and fat replacer. Notably, the glycemic content of the product was limited via the incorporation of the natural sweetener stevia. Furthermore, V3 displayed a potent free radical scavenging and reduction potential, which may also be attributed to the presence of the studied phytochemicals. Noteworthy, no significant microbial growth occurred within 20 days (4°C) of the development of the product in the absence of any additives or preservatives, and the cost of the product was found to be

comparable to most chocolates available commercially. Interestingly, in contrast to earlier reports regarding the development and analysis of prebiotic chocolates, the present study estimated the functional properties of the developed prebiotic chocolate, namely, antioxidant and prebiotic potential, in a simulated *in vitro* human intestinal environment to estimate the effectiveness of the product after its consumption by human beings. Noteworthy, V3 was observed to considerably manifest its antioxidant ($81.21\pm0.8\%$ DPPH inhibition and 2.08 ± 0.01 μg/mL reducing power) and prebiotic (OD 0.50 at 20 hours) actions even after *in vitro* simulated human intestinal digestion compared to S. Thus, the developed prebiotic and antioxidant-rich chocolates may serve as a healthy alternative compared to their conventional counterparts, which may be accommodated by the population for improved health outcomes owing to their nutritional and functional effects along with substantial maintenance of the functional parameters after an *in vitro* intestinal digestion. Nevertheless, estimation of the functionality of the developed products in an *in vivo* system may yield improved interpretations and enhance the scope of application of the prepared products for human use, thereby indicating the future prospects of the current study.

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