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RESEARCH ARTICLE

Shelf Life and Physicochemical Attributes Evaluation of Dark Chocolate Made with Putative Probiotic *Lactococcus lactis* sub sp. lactis Isolated from Fermented *Theobroma cacao L.* Fruit

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Abstract

Objectives: To develop a probiotic dark chocolate by incorporating *Lactococcus lactis* sub sp. *lactis*, and evaluate its physicochemical and sensory characteristics, probiotic viability during storage, and behavior during *in vitro* gastrointestinal digestion. **Methods:** The probiotic dark chocolate was formulated through the inclusion of a strain of *Lactococcus lactis* subsp. *lactis*, sourced from the fermented *Theobroma cacao L. forastero* variety fruit harvested in Navsari, Gujarat, India. Subsequently, this probiotic dark chocolate underwent storage at both 4°C and 25°C for 90 days. In parallel, a control dark chocolate, devoid of probiotics, was subjected to testing. Analytical assessments encompassed parameters such as viscosity, pH, water activity, sensory evaluation, and probiotic viability evaluations under simulated gastric and pancreatic conditions. **Findings:** After 90 days of storage at 4°C, *Lactococcus lactis* subsp. *lactis* in probiotic dark chocolate declined from 8.25 to 6.42 log CFU/g (79.75% survival, $p \leq 0.05$), offering potential health benefits. pH shifted from 5.81 to 5.87 ($p \leq 0.05$), water activity increased (0.42 to 0.52, $p \leq 0.05$), and viscosity(cP) decreased from 878.01 to 651.00 ($p \leq 0.05$). The dark chocolate matrix improved probiotic viability in the gastrointestinal tract compared to free cells. Sensory attributes remained unaffected. Dark chocolate shows promise as a probiotic delivery system. **Novelty:** This study unveils the potential of an unexplored lactic acid bacteria strain from *Theobroma cacao L.* beans in Navsari, Gujarat, India. The novelty lies in successfully incorporating *Lactococcus lactis* subsp. *lactis* into dark chocolate, showing high viability and comparable characteristics to control chocolate. The dark chocolate matrix proves to be an effective probiotic delivery system.

Keywords: *Lactococcus lactis* subsp. *lactis*; *Theobroma cacao* L; Probiotic Product Development; Viability During Storage; Probiotic Delivery System

1 Introduction

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defined probiotics in October 2013. According to ISAPP, probiotics are illustrated as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." This definition emphasizes the requirement for viable microorganisms and the potential positive effects they can have on the individual consuming them [(1)]. Probiotic foods have garnered significant attention within the sphere of functional foods due to their capacity for enhancing gut microbiota balance, boosting the immune system, and promoting overall well-being. The growing interest in probiotic products, which encompasses yoghurt, fermented meals, beverages, and dietary supplements, reflects their perceived benefits. This increased consumer awareness has led to a surge in demand for probiotic-functional foods [(2)].

In the contemporary landscape of functional foods, especially in the realm of probiotic supplements, the global market has witnessed rapid expansion. Current trends in the consumption of probiotics are associated with increased levels of health consciousness and the availability of probiotics in the form of dietary supplements [(3)]. The growth trajectory of the global probiotics market presents an intriguing perspective when connected with the Indian probiotics market. As per the Global Probiotic Market Outlook, 2028, there is a substantial potential for significant growth in the global market. Projections indicate that by 2028, the global probiotics market is poised to reach an impressive valuation of USD 105.27 billion, marking a noteworthy increase from its initial valuation of USD 64.52 billion recorded in 2022. This anticipated expansion is expected to be characterized by the compound annual growth rate (CAGR) of 8.69% spanning the years between 2023 and 2028 [(4)]. Simultaneously, the Indian probiotics market also shows promising growth. In 2021, it was valued at INR 2.6 billion, and according to projections by the IMARC (International Market Analysis Research and Consulting) Group, it is expected to attain a valuation of INR 7.7 billion by 2027. This projection suggests an expected CAGR of approximately 20.50% during the period spanning from 2022 to 2027 [(5)]. These two data points illustrate that while the global probiotics market is experiencing steady growth with a CAGR of 8.69%, the Indian probiotics market is on a more rapid growth trajectory with a higher CAGR of approximately 20.50% during the same period. This contrast highlights the robust potential for the probiotics industry in India and its contribution to the overall expansion of the global probiotics market.

The history of chocolate has its origins dating back over 4,000 years, with its initial discovery in what is now modern-day Mexico, where cocoa bushes were first found. The production of chocolate begins with cocoa beans, from which cocoa powder and cocoa butter are initially extracted, serving as their fundamental ingredients. Notably, cocoa butter is rich in various fatty acids, encompassing both saturated and unsaturated types, free fatty acids, antioxidants, mineral salts, and vitamins. Furthermore, chocolate serves as a source of tryptophan, serotonin, and dopamine, and it positively impacts cerebral metabolic processes while stimulating endorphin production [(6)]. Considering these attributes, probiotic chocolates emerge as intriguing products that align with the criteria of functional foods, being rich in nutrients and health-promoting compounds, thus meeting the expectations of consumers, especially children. Currently, there are relatively few recognized high-quality chocolate products that possess both pro- and prebiotic properties. Therefore, there is a pressing need for the development of novel chocolate products designed to be used as functional foods. This need is particularly

pertinent given the prevalence of infectious diarrhea, a significant issue among adults who travel frequently (commonly referred to as traveler's diarrhea), as well as among individuals with compromised immune systems, such as HIV carriers, the elderly, and those undergoing chemotherapy[7].

Chocolate has been identified as an effective carrier for probiotic strains such as Klindt-Toldam et al. (2016) have identified chocolate as an effective carrier for probiotic strains like *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* HN019[8]. Islam et al. (2022) have also investigated the use of chocolate as a carrier, particularly with *Lactobacillus acidophilus* LDMB-01[9]. Additionally, Mirković et al. (2018) have explored the potential of dark chocolate as a protective medium for *Lactobacillus plantarum* in previous research studies[10]. These studies have shown that chocolates serve as an excellent protective medium, shielding the bacteria from the harsh conditions of the gastrointestinal tract. Moreover, different varieties of chocolate exhibit the potential to function as matrices for probiotics, offering opportunities for the development of novel functional foods in the future.

To the best of the author's knowledge, there is limited information available regarding the impact on probiotic viability after exposure to digestion when incorporated into dark chocolate. Consequently, the inclusion of novel probiotic cells, specifically *Lactococcus lactis* subsp. *lactis*, derived from *Theobroma cacao L.* forastero variety in the Navsari region of Gujarat, India, into dark chocolate without encapsulation represents an innovative research direction. The hypothesis posited that the viability of probiotic cells within dark chocolate could potentially be enhanced during both shelf storage and under conditions simulating gastrointestinal digestion. Hence, in consideration of the existing knowledge gaps, this study was undertaken to formulate probiotic dark chocolate. The primary objective was to assess the viability of the novel probiotic strain, *Lactococcus lactis* subsp. *lactis*, when integrated into the dark chocolate matrix. This assessment encompassed multiple facets, including sensory tests to evaluate visual appearance, color, flavor, texture, and overall acceptability, as well as a thorough examination of physicochemical attributes such as available water content, pH, and viscosity. Additionally, these analyses were conducted over two key phases: during the storage period and through the application of an *in vitro* gastrointestinal digestion model. This comprehensive approach allowed for a holistic understanding of the potential of probiotic dark chocolate as a functional food product.

2 Methodology

2.1 *Lactococcus lactis* subsp *lactis* isolation using cocoa beans

With the objective of initiating the process of isolating *Lactococcus lactis* subsp. *lactis* from fermented forastero variety of *Theobroma cacao L.* fruit derived from Navsari, Gujarat, India (20°55'38"N,72°53'54"E), 50 g of cocoa beans and pulp were combined with 450 mL of water containing 0.1% peptone. The total number of live bacterial cells has been quantified using the pour plate technique. With 0.1% peptone water, the rinsed water was serially diluted. Then, 100 μL of diluted samples were transferred to de Man, Rogosa, and Sharpe (MRS, Himedia, Mumbai, India) agar plates supplemented with 0.1% $CaCO_3$ [11]. For 24-48 hours, the plates were incubated at 37 °C. Counted visible cells were converted to CFU/ml[12].

2.2 Incorporation of *Lactococcus lactis* subsp *lactis* in dark chocolate

A cell pellet was produced in the experiment by using a culture of *Lactococcus lactis* subsp. *lactis* with an initial concentration of 8.25 log CFU/g. At 25 °C for 15 minutes, the culture was centrifuged at 2600*g. A pellet of bacterial cells has been generated as a result of the centrifugation process that separated the bacterial cells from the liquid media. 100 grams of dark chocolate, subjected to a temperature of 40 °C, were employed after extracting the cell pellet. The melted chocolate was then subjected to a tempering procedure, which involves cooling and reheating to achieve appropriate crystal formation and stability. The tempering process involved cooling the chocolate to 27 °C and then reheating it for 1-2 minutes at 32 °C. Proper stirring was maintained during this procedure to ensure equal dispersion and incorporation of the cell pellet. After thoroughly blending the chocolate and cell combination, it was transferred into the appropriate moulds. After that, the moulds were stored at 4 °C for 30 minutes to allow the chocolate to set and solidify. After the solidification process, the chocolates were carefully de-moulded by hand. They were then covered with 2 mm-thick aluminum foil and stored at a temperature of 4 °C and 25 °C. This temperature was chosen to maintain the stability and quality of the chocolates during storage.

2.3 Viability of *Lactococcus lactis* subsp *lactis* in dark chocolate during storage

Employing the total plate count technique, the viability of *Lactococcus lactis* subsp. *lactis* in dark chocolate was assessed during 90 days of storage at intervals of 7 days. A serial dilution was prepared by first melting 5 g of the chocolate sample at 37 °C and blending it with 45 mL of buffered peptone water. 100 μL of the suspension was applied to MRS agar, which was then incubated at 37 °C for 24-48 hours. The number of colonies was enumerated and represented as log CFU/g. For probiotic dark chocolate,

the percentage of live bacteria was determined.

2.4 Storage study

In order to analyse alterations in probiotic viability at intervals of every 7 days for up to 90 days of storage, both control and probiotic dark chocolates were preserved by wrapping in a foil made of aluminum with a minimum thickness of 0.2 mm at 25 °C and 4 °C. Additionally assessed were the physicochemical attributes of dark chocolate with *Lactococcus lactis* subsp. *lactis*.

2.5 Physicochemical analysis

The mentioned study aimed to scrutinize the physicochemical attributes of probiotic dark chocolate over a protracted 90-day storage period, under controlled conditions at 4 °C. A comparative analysis was conducted with conventional dark chocolate, devoid of probiotic elements. The investigation meticulously assessed three key parameters: pH, water activity, and viscosity, employing specialized instrumentation. The physicochemical analyses were conducted in accordance with the methodology described by Islam et al., with slight modifications [9].

2.5.1 pH Measurement

Employing a digital pH meter, the pH of the chocolates was determined. Throughout the 90-day storage period, the pH levels of the probiotic and control chocolates were monitored every 7 days. This made it possible for researchers to remain vigilant for any possible fluctuations in the acidity or alkalinity of the chocolates, which might have an influence on their overall stability and quality.

2.5.2 Water Activity Assessment

In addition to pH, the water activity of the chocolates was also measured. Water activity refers to the quantity of water that is accessible for microbial growth and chemical reactions. It is a crucial parameter for determining the shelf stability and microbial safety of food products. A water activity meter (Maxtech, Mumbai, India) set at 27±1 °C was employed to measure the water activity of both chocolate samples. Measurements were obtained at intervals of every 7 days throughout the 90-day storage period.

2.5.3 Viscosity Analysis

Viscosity analyses were systematically conducted on both probiotic dark chocolate and control dark chocolate samples at seven-day intervals throughout a 90-day storage period. A precision viscometer (Thermo Fisher Scientific in Mumbai, India) was employed for this purpose. Prior to viscosity measurements, the chocolate samples were subjected to incubation at a controlled temperature of 37 °C for one hour. To attain precise and reliable measurements, a deliberate interval was observed, ensuring that five complete rotations were completed before recording the viscosity data.

The overarching goal of this study was to assess the enduring quality and stability of probiotic dark chocolate. This assessment hinged on the systematic monitoring of pH, water activity, and viscosity. These metrics were selected to enable forecasts concerning alterations in acidity, moisture content, and microbiological safety throughout the prolonged storage period. Importantly, it should be noted that the experimental design included a control group comprising standard dark chocolate devoid of live cells. This control group served as a reference point for contrasting the physicochemical attributes and quality between probiotic dark chocolate and its conventional counterpart. All procedural steps described herein were meticulously executed under aseptic conditions, safeguarding the integrity of the experiment and minimizing the potential for contamination.

2.6 The viability of probiotics during *in vitro* gastrointestinal digestion

The approach described by Brodkorb et al., was used to evaluate the cell viability of a probiotic strain, *Lactococcus lactis* subsp. *lactis*, during the gastrointestinal digestion of probiotic dark chocolate and control dark chocolate. The assessment process includes two key steps: simulating the stomach phase using simulated gastric fluid (SGF) and simulating the intestinal phase using an intestinal juice solution [13]. Five parts of chocolate and four parts of SGF were mixed to make the final chocolate sample and SGF combination. Double-distilled water was then added, one part at a time, to the mixture. Once the quantity of porcine pepsin reached 2000 U/mL, $CaCl_2$ was added until it attained a 0.070 mM concentration. Using 1 M HCl, the mixture's pH was brought down to 3.0. The pH of the mixture was adjusted to 3.0 using 1 M HCl. The resulting mixture was then incubated for two hours at 37 °C to simulate the gastric phase of digestion.

The Kazancıgil et al., method was employed to assess the cell viability of *Lactococcus lactis* subsp. *lactis* in response to intestinal juice tolerance. The method involved the following steps:

1. **Preparation of a sterile solution:** A sterile solution was prepared by combining 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride in 100 mL of distilled water. Add 0.1 g of trypsin and 1.8 g of bile salts.
2. **Adjustment of pH:** The pH of the solution was raised to 8.0 using 0.5 M sodium hydroxide.
3. **Sterilization process:** The simulated gastric and intestinal juices were sterilized by filtration through a 0.45 μm membrane to remove any potential contaminants.
4. **Fermentation and centrifugation:** The chocolate samples, after undergoing the gastric phase simulation, were fermented and then centrifuged at 500 * g for 5 minutes.
5. **Washing of the pellet:** The resulting pellet obtained from centrifugation was washed three times with phosphate-buffered saline (PBS) at neutral pH. This step helps remove residual digestion fluid and other contaminants.
6. **Incubation:** The washed sample was incubated with the prepared intestinal juice solution. The incubation was performed for 4 hours at 37°C in a shaking incubator.
7. **Dilution and plating:** After incubation, aliquots of 1 mL from each incubation fluid were obtained. These aliquots were then diluted with 9 mL of 0.2 M sterile phosphate buffer at neutral pH. The diluted samples were plated on MRS agar medium in triplicate.
8. **Colony counting and viability calculation:** The plates were incubated, and after an appropriate incubation period, the resulting colonies were counted. The colony-forming units per milliliter (CFU/mL) were calculated based on the dilution factor to determine the cell viability [14,15].

Employing this approach, the viability of *Lactococcus lactis* subsp. *lactis* during the simulated gastrointestinal digestion process could be evaluated. Researchers might ascertain the capacity of the probiotic strain to endure digestive difficulties and perhaps impart its positive benefits to the consumer by assessing the survival and development of the probiotic strain under these circumstances.

2.7 Microbiological analysis

Additional microbiological studies on the probiotic dark chocolate were carried out to evaluate the hygienic state across the product manufacturing method. The following counts were carried out:

1. **On MRS agar, viable lactic acid bacteria were counted:** The lactic acid bacteria-selective MRS agar was used for plating the probiotic dark chocolate samples. The colonies were counted after being incubated at the proper time and temperature (often 37 °C) for the necessary duration of time (for example, 24–48 hours). The quantity of viable lactic acid bacteria in the dark chocolate may be determined by this count [16].
2. **Coliform count on Violet Red Bile Agar (VRBA):** The chocolate samples were plated on VRBA, a dual-purpose medium for coliforms, to ascertain the presence of coliform bacteria. The colonies were counted after incubation under aerobic conditions at the designated temperature (often 37 °C) for 24–48 hours. The amount of probable faecal contamination in the product can be determined by the coliform count [17].
3. **Yeast and mould count on Potato Dextrose Agar (PDA):** On Potato Dextrose Agar (PDA), a medium that is suitable for the development of yeasts and moulds, chocolate samples were placed for yeast and mould counting. The plates were incubated for five days in aerobic conditions at the designated temperature (typically 28 °C). Yeast and mould colonies were counted after incubation to determine the quantity of these microorganisms in the final product [18].

These extra microbiological tests can be used to assess the overall hygienic status of probiotic dark chocolate throughout the manufacturing procedure. The MRS agar viable lactic acid bacteria count aids in determining the viability of a probiotic strain. The coliform count on VRBA suggests that faecal contamination may be present, while the yeast and mould count on PDA indicates the amount of yeast and mould that exists within the product. These counts help ensure the safety and quality of the probiotic dark chocolate.

2.8 Sensory evaluation

The sensory analysis of dark chocolates encompassed a comprehensive assessment of attributes encompassing color, appearance, texture, flavor, and overall acceptability. The evaluative endeavor encompassed two distinct categories: control chocolates and

chocolates supplemented with probiotics. The sensory appraisal was executed by a cohort of 25 individuals, specifically chosen based on their affinity for chocolate and falling within the age bracket of 18 to 30 years. The participant pool encompassed both academic personnel and students in Ahmedabad. To facilitate the sensory evaluation process, a structured approach was adopted. The evaluative schema involved the utilization of a 9-point hedonic scale, wherein a rating of 9 corresponded to an extreme liking, a rating of 5 indicated a neutral stance of neither preference nor aversion, and a rating of 1 signified an extreme disliking. To ensure a neutral sensory baseline, participants were directed to rinse their oral cavities with water before each tasting the sample [19].

2.9 Ethical statement

The individuals comprising the sensory panel provided their verbal consent to partake in the research initiative. The findings, as delineated in the manuscript, are presented holistically, devoid of individual responses from the panelists. Importantly, the outcomes encapsulated within the study do not divulge any form of sensitive or personal particulars pertaining to the participants engaged in the sensory panel. Thus, the necessity for a written formal consent declaration was deemed unnecessary for both the execution of the investigation and the subsequent dissemination of its outcomes. It is noteworthy that this consent verification was corroborated by two researchers conducting the study, and such confirmation was duly documented within the laboratory journal.

2.10 Statistical evaluation

The acquired data were rendered in the form of the mean, derived from three individual replicates (n = 3), along with their corresponding standard deviations. To discern notable distinctions ($p < 0.05$) among the data sets, an analysis of variance (ANOVA) was executed, employing the SPSS program version 29.0.1.0 (SPSS Inc., USA). After this, to establish a hierarchical ranking of the discernibly distinct groupings, Duncan's multiple range tests were deployed.

3 Results and discussion

3.1 Viability of *Lactococcus lactis* subsp *lactis* in dark chocolates during storage

The viability of *Lactococcus lactis* subsp. *lactis*, a probiotic bacterium, in dark chocolates decreased over time during storage. The dramatic decrease in cell viability was more pronounced at higher temperatures, particularly at 25 °C. After 90 days of storage at 25 °C, the number of *Lactococcus lactis* subsp. *lactis* cells in the dark chocolate decreased to 0.2 CFU/g from 8.25 log CFU/g ($p \leq 0.05$) as illustrated in Figure 1. The research of Champagne et al., which also noted a similar pattern in the survivability of immobilised probiotics in different chocolate matrices, is compatible with the findings reported in the current research [20]. There are several factors that can contribute to the diminished viability of probiotic bacteria in chocolate. Exposure to oxygen, a decrease in pH, and the metabolic activity of lactic acid can all play a vital role in reducing the survival of probiotics. These factors may interact with each other and the chocolate matrix to affect the viability of the bacteria over time [21].

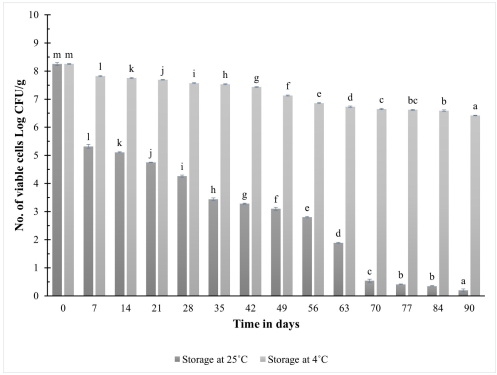

Fig 1. *Lactococcus lactis* subsp. *lactis* viability in dark chocolate during 90 days of storage at 25°C and 4°C in log CFU/g. The values depict the mean ± SD of three replicate assays. Statistical analysis (One-way ANOVA and Duncan's multiple range tests) identifies significant differences ($p \leq 0.05$) marked by letters (a–m).

3.2 Physicochemical properties

Table 1 presents the physicochemical characteristics of both the control dark chocolate and probiotic chocolates containing *Lactococcus lactis* subsp. *lactis* during refrigerated storage at 4°C for up to 90 days. The finding suggests that there were no discernible variations in the physicochemical characteristics between the control dark chocolates and the probiotic dark chocolates during this refrigerated storage period. These findings align with a previous study conducted by Kvakova et al., which likely investigated the incorporation of probiotics in dark chocolate. This previous study likely had similar results, demonstrating that the integration of probiotics did not significantly impact the physicochemical characteristics of dark chocolate during refrigerated storage[22].

Dark chocolate containing *Lactococcus lactis* subsp. *lactis* had an initial pH of 5.81 and climbed to 5.87 over 90 days of storage, while the pH of control dark chocolate increased insignificantly (Table 1). Probiotics can develop in this pH range since it favours a neutral pH. During the 90 days of storage, there was a noticeably greater pH change in the dark chocolate containing *Lactococcus lactis* subsp. *lactis* than in dark chocolate used as a control.

Water activity (a_w) for the control dark chocolate ranged from 0.39 to 0.49 throughout the 90-day storage period, while it ranged from 0.42 to 0.52 for the dark chocolate containing *Lactococcus lactis* subsp. *lactis* (Table 1). This outcome was anticipated given the refrigerated storage conditions implemented. In fact, the majority of studies advise freezing or refrigeration for probiotic preservation[23,24]. An essential factor that can influence the viability of probiotics in a food matrix is water activity (a_w). Water activity refers to the availability of water molecules for microbial growth and biochemical reactions. The water activity of a food product can affect the survival and growth of microorganisms, including probiotics. If the water activity is too low, microbial activity may be limited due to water scarcity. Conversely, if the water activity is too high, it can lead to microbial proliferation and potentially spoilage[25]. Even though both chocolate samples in the current investigation had water activity (a_w) values below 0.52, the meagre rise in water activity (a_w) following storage for 90 days ($p \leq 0.05$) may indicate the microbiological safety of chocolate since few microorganisms may grow in such an environment. When compared to probiotic chocolate, the control chocolate had lower levels of a_w, which might be explained by the use of bacterial pellets that were extracted from a liquid medium.

The fact that the present findings concur with those of Champagne et al., shows that probiotic stability within the chocolate matrix is trending in a similar direction. This reinforces the notion that the chocolate matrix, whether it is semisweet or dark chocolate, plays a role in maintaining the viability of probiotic bacteria[20].

In the study, an overall observation revealed that the disparity in viscosity between the control dark chocolate and probiotic dark chocolate diminished over an extended storage period of 90 days. Specifically, as the duration of storage progressed, the viscosity of the probiotic chocolate exhibited a gradual reduction ranging from 878.01 to 651.00 cP ($p \leq 0.05$) as described in Table 1. Conversely, the viscosity of the control dark chocolate samples displayed an incremental trend, with a slight increase observed up to the 90-day storage mark. These findings align with outcomes reported by Foong et al., who investigated the inclusion of probiotics in dark chocolate[26].

Table 1. pH, Water Activity (aW), and Viscosity (cP) changes in control and probiotic dark chocolate during 90-day refrigerated storage at 4°C, evaluated at 37°C

Days of incubation at 4°C	Value of pH		Value of Available water (aW)		Value of viscosity (cP)	
	Control dark chocolate	Probiotic Dark chocolate	Control dark chocolate	Probiotic Dark chocolate	Control dark chocolate	Probiotic Dark chocolate
0	5.79 ± 0.03^{a}	5.81 ± 0.03^{a}	0.39 ± 0.01^{a}	0.42 ± 0.01^{a}	529.67 ± 0.57^{a}	878.01 ± 1.00^{m}
7	5.85 ± 0.02^{a}	5.82 ± 0.03^{ab}	0.39 ± 0.02^{a}	0.42 ± 0.01^{a}	541.00 ± 1.00^{b}	863.01 ± 1.00^{l}
14	5.87 ± 0.03^{a}	5.83 ± 0.02^{ab}	0.39 ± 0.03^{a}	0.43 ± 0.05^{ab}	546.00 ± 2.64^{c}	855.03 ± 2.64^{k}
21	5.93 ± 0.04^{ab}	5.84 ± 0.03^{ab}	0.40 ± 0.02^{a}	0.44 ± 0.01^{abc}	602.00 ± 1.00^{d}	853.06 ± 2.00^{k}
28	5.91 ± 0.03^{a}	5.84 ± 0.02^{ab}	0.40 ± 0.01^{a}	0.46 ± 0.01^{bcd}	608.33 ± 1.52^{e}	832.67 ± 2.08^{j}
35	5.95 ± 0.07^{ab}	5.84 ± 0.02^{ab}	0.41 ± 0.02^{ab}	0.46 ± 0.01^{cd}	610.67 ± 0.58^{f}	781.67 ± 1.52^{i}
42	5.99 ± 0.05^{ab}	5.85 ± 0.04^{ab}	0.41 ± 0.02^{ab}	0.47 ± 0.02^{cd}	611.67 ± 1.52^{f}	772.67 ± 2.51^{h}
49	6.13 ± 0.05^{bc}	5.86 ± 0.04^{ab}	0.42 ± 0.03^{ab}	0.47 ± 0.02^{cd}	614.34 ± 1.15^{g}	756.34 ± 3.06^{g}
56	6.22 ± 0.05^{c}	5.86 ± 0.03^{ab}	0.43 ± 0.02^{abc}	0.48 ± 0.01^{de}	618.00 ± 1.00^{h}	718.33 ± 2.08^{f}
63	6.27 ± 0.02^{c}	5.86 ± 0.02^{ab}	0.45 ± 0.01^{bcd}	0.49 ± 0.01^{def}	620.01 ± 1.00^{h}	700.33 ± 20.8^{e}
70	6.31 ± 0.03^{c}	5.86 ± 0.03^{ab}	0.46 ± 0.03^{cde}	0.51 ± 0.03^{efg}	623.34 ± 0.58^{i}	691.00 ± 2.00^{d}
77	6.33 ± 0.02^{c}	5.86 ± 0.02^{ab}	0.47 ± 0.01^{de}	$0.51\pm0.02f^{g}$	625.33 ± 0.62^{i}	673.34 ± 2.52^{c}
84	6.38 ± 0.03^{d}	5.87 ± 0.01^{b}	0.47 ± 0.02^{cde}	0.52 ± 0.02^{g}	627.67 ± 0.58^{j}	633.66 ± 1.53^{b}
90	6.40 ± 0.01^{d}	5.87 ± 0.02^{b}	0.49 ± 0.01^{e}	0.52 ± 0.01^{g}	631.67 ± 1.52^{k}	651.00 ± 2.01^{a}

Continued on next page

Table 1 continued

Dark chocolate was formulated with *Lactococcus lactis* subsp. *lactis* as a probiotic component, whereas the control chocolate was prepared without the incorporation of any probiotics. The values depict the mean ± SD of three replicate assays. Statistical analysis (One-way ANOVA and Duncan's multiple range tests) identifies significant differences ($p \leq 0.05$) marked by letters (a–e).

3.3 Probiotic viability during *in vitro* gastrointestinal digestion

The potential of *Lactococcus lactis* subsp. *lactis* to survive under stimulated gastrointestinal circumstances has been investigated in both free cells and the dark chocolate matrix (Figure 2) At concentrations of 8.25 log CFU/mL, the free cell and matrix were both first introduced into a simulated gastric fluid. After 2 hours of incubation, all of the *Lactococcus lactis* subsp. lactis cell numbers decreased drastically, and after 4 hours, all of the *Lactococcus lactis* subsp. *lactis* cells had been killed. This was due to the fact that the number of *Lactococcus lactis* subsp. *lactis* cells that survived in the dark chocolate matrix rapidly decreased as the incubation duration increased ($p \leq 0.05$). The direct impact of *in vitro* gastrointestinal variables such as acidity, enzymes, incubation period, and oxygen level may be contributing factors [27].

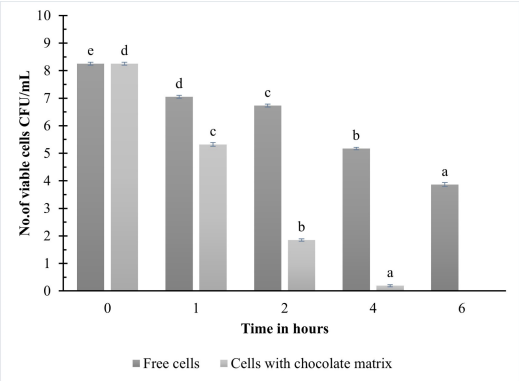

Fig 2. *Lactococcus lactis* subsp. *lactis* survival ability in dark chocolate during exposure to the gastric and intestinal environment in log CFU/ml. The values depict the mean ± SD of three replicate assays. Statistical analysis (One-way ANOVA and Duncan's multiple range tests) identifies significant differences ($p \leq 0.05$) marked by letters (a–e)

Lactococcus lactis subsp. *lactis* with dark chocolate matrix was discovered to have adequate survival despite receiving exposure to the gastrointestinal environment of a stimulated stomach for 2 hours. These *Lactococcus lactis* subsp. *lactis* cells were then incubated for an additional 4 hours in the stimulated small intestine fluid, and they continued to be viable up to 5.17 log CFU/mL at 4 hours and 3.84 log CFU/mL at 6 hours. Probiotics may receive extra protection from dark chocolate matrices while they transit across the challenging digestive system environment. The higher vitality of dark chocolate matrix cells in comparison to free cells can be attributed to the matrix's protective properties against adverse circumstances in the current study [9].

As a consequence, it was discovered in the present research that dark chocolate matrix is an excellent transport medium for safeguarding probiotics from the gastrointestinal environment, enabling a high and adequate survival rate for human health.

3.4 Microbiological analysis

The viability of free and encapsulated *Lactococcus lactis* subsp. *lactis* was maintained up to 6.42 log CFU/mL for 90 days at refrigerated temperature (4 °C). However, after 90 days of storage at 25°C, the *Lactococcus lactis* subsp. *lactis* counts in dark chocolate with free and encapsulated *Lactococcus lactis* subsp. *lactis* respectively, drastically decreased from 8.25 log CFU/g. The conclusions are consistent with those reported by Sharouba et al., Mould, yeast, and coliforms were not present in produced or preserved samples, indicating that hygiene standards and surroundings were upheld both throughout preparation and the entire storage time [28].

3.5 Sensory evaluation

In the context of the sensory evaluation of dark chocolate infused with probiotics, both control and experimental samples stored at a controlled temperature of 4°C were subjected to comprehensive sensory assessments. These evaluations were carried

out over 90 days, during which the probiotic-infused samples exhibited consistent viable microbial counts of up to 6.42 log CFU/g. A test panel comprising 50 untrained individuals participated in the sensory analysis, evaluating key attributes such as visual appearance, color, flavor, texture, and overall acceptability, as elucidated in the provided tabular representation (Table 2). Upon initial analysis at day 0, no perceptible disparities in sensory scores were discerned between the control and probiotic-enriched dark chocolate variants. Over the extended storage period of 90 days, both cohorts of samples demonstrated sustained uniformity in terms of color and visual attributes. Notably, akin flavor profiles were noted during the initial 30-day storage interval, followed by a modest attenuation by day 90. In contrast, the textural attributes exhibited a discernible reduction compared to their freshly prepared counterparts, a divergence that achieved statistical significance ($p \leq 0.05$) within the initial 30-day timeframe and further escalated by the conclusion of the 90-day storage duration. However, despite these observable textural alterations, the cumulative sensory analysis yielded consistently favorable scores in terms of overall liking.

Table 2. Sensory evaluation of control and probiotic dark chocolates containing *Lactococcus lactis* subsp. *lactis* stored at 4 °C

Sample	Color	Appearance	Flavor	Texture	Overall acceptability
Probiotic dark chocolate at Day 0	8.40 ± 0.51^{a}	8.55 ± 0.61^{a}	8.17 ± 0.50^{a}	8.36 ± 0.12^{a}	8.66 ± 0.67^{a}
Control dark chocolate at Day 0	8.36 ± 0.33^{a}	8.49 ± 0.21^{a}	8.16 ± 0.56^{a}	8.38 ± 0.11^{a}	8.61 ± 0.52^{a}
Probiotic dark chocolate at Day 30	8.15 ± 0.19^{a}	8.24 ± 0.42^{a}	8.05 ± 0.58^{a}	7.05 ± 0.03^{b}	8.06 ± 0.18^{a}
Control dark chocolate at Day 30	8.16 ± 0.17^{a}	8.25 ± 0.42^{a}	8.06 ± 0.58^{a}	7.17 ± 0.03^{b}	8.16 ± 0.18^{a}
Probiotic dark chocolate at Day 90	8.10 ± 0.34^{a}	8.21 ± 0.93^{a}	7.05 ± 0.34^{b}	6.80 ± 0.31^{b}	7.46 ± 0.11^{b}
Control dark chocolate at Day 90	8.08 ± 0.33^{a}	8.19 ± 0.93^{a}	7.26 ± 0.22^{b}	6.38 ± 0.31^{c}	7.38 ± 0.41^{b}

The values depict the mean ± SD of three replicate assays. Statistical analysis (One-way ANOVA and Duncan's multiple range tests) identifies significant differences ($p \leq 0.05$) marked by letters (a–e).

Notably, the introduction of *Lactococcus lactis* subsp. *lactis* into the dark chocolate matrix, as demonstrated in this study, did not result in significant alterations to the sensory characteristics when compared to the control group. This alignment with previous research findings, exemplified by studies such as those conducted by Islam et al., in which chocolate formulations enriched with *Lactobacillus acidophilus* LDMB-01 displayed sensory attributes closely resembling their non-inoculated counterparts, reinforces the consistency between our observations and well-established scientific discourse[9].

This groundbreaking study introduces novel insights into probiotic *Lactococcus lactis* subsp. *lactis* incorporation within dark chocolate matrices, utilizing dark chocolate prepared from fermented beans of *Theobroma cacao L.* plants isolated from Navsari, Gujarat, India, for the first time. In comparison with other probiotic-rich foods like yoghurt, fermented beverages, and milk chocolate, this uniquely sourced dark chocolate emerges as the superior carrier derived from Navsari, Gujarat, due to its temperature-dependent viability patterns, protective qualities during simulated gastrointestinal digestion, and consistent physicochemical attributes during refrigerated storage, ensuring reliability. Microbiological analysis confirms sustained viability and safety. The matrix's significant influence on probiotic viability aligns with prior research, underscoring its role in maintenance. Moreover, the dark chocolate matrix emerges as a potent transport medium, preserving probiotics within the gastrointestinal environment. These findings highlight the distinct advantages of dark chocolate and its potential implications for enhanced probiotic delivery systems.

4 Conclusion

Probiotic viability during storage and an in vitro model of gastrointestinal digestion were both investigated after *Lactococcus lactis* subsp. *lactis* were introduced to dark chocolate which was successfully isolated from fermented beans of *Theobroma cacao L.* The inclusion of possible probiotics had no impact on the physicochemical and sensory properties when compared with control dark chocolate. The potential probiotic *Lactococcus lactis* subsp. *lactis* in dark chocolate remains viable during 90 days of storage and *in vitro* gastrointestinal digestion. On the contrary, the number of cells that did not adhere to the matrix was significantly reduced, and these cells often vanished following digestion. Probiotic dark chocolates additionally demonstrate this product's potential for implementation in the marketing of functional foods. Prior to the manufacturing of probiotic chocolate on a commercial scale, however, more modifications and enhancements are required. This study suggests that dark chocolate

may function as a probiotic carrier for *Lactococcus lactis* subsp. *lactis* with satisfactory viability during storage and digestion in SGF. Future production of probiotic dark chocolate on a small or medium scale can be accomplished using the method presented here.

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References

1) Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nature Reviews Gastroenterology & Hepatology*. 2021;18(9):649–667. Available from: https://doi.org/10.1038/s41575-021-00440-6.
2) Yilmaz-Ersan L, Ozcan T, Akpinar-Bayizit A. Assessment of socio-demographic factors, health status and the knowledge on probiotic dairy products. *Food Science and Human Wellness*. 2020;9(3):272–279. Available from: https://doi.org/10.1016/j.fshw.2020.05.004.
3) Chugh B, Kamal-Eldin A. Bioactive compounds produced by probiotics in food products. *Current Opinion in Food Science*. 2020;32:76–82. Available from: https://doi.org/10.1016/j.cofs.2020.02.003.
4) Probiotics Market in India to Hit INR 7.7 billion by 2027 at CAGR of 20.50%, IMARC Group. 2023. Available from: https://www.openpr.com/news/2922905/probiotics-market-in-india-to-hit-inr-7-7-billion-by-2027-at-cagr.
5) Global Probiotics Market Outlook. 2023. Available from: https://www.giiresearch.com/report/bon1302979-global-probiotics-marketoutlook.html.
6) Cielecka-Piontek J, Dziedziński M, Szczepaniak O, Kobus-Cisowska J, Telichowska A, Szymanowska D. Survival of commercial probiotic strains and their effect on dark chocolate synbiotic snack with raspberry content during the storage and after simulated digestion. *Electronic Journal of Biotechnology*. 2020;48:62–71. Available from: https://doi.org/10.1016/j.ejbt.2020.09.005.
7) Bloomfield SF, Ackerley L, Bockmühl D, Signorelli C, Münter L, Langsrud S, et al. Containing the burden of infectious diseases is everyone's responsibility: A call for an integrated strategy for developing and promoting hygiene behaviour change in home and everyday life. 2018. Available from: https://www.ifh-homehygiene.org/review/containing-burden-infectious-diseases-everyones-responsibility-call-integrated-strategy.
8) Klindt-Toldam S, Larsen SK, Saaby L, Olsen LR, Svenstrup G, Müllertz A, et al. Survival of Lactobacillus acidophilus NCFM® and Bifidobacterium lactis HN019 encapsulated in chocolate during in vitro simulated passage of the upper gastrointestinal tract. *LWT*. 2016;74:404–410. Available from: https://doi.org/10.1016/j.lwt.2016.07.053.
9) Islam MZ, Masum AKM, Harun-Ur-Rashid MD. Milk chocolate matrix as a carrier of novel Lactobacillus acidophilus LDMB-01: Physicochemical analysis, probiotic storage stability and in vitro gastrointestinal digestion. *Journal of Agriculture and Food Research*. 2022;7:100263. Available from: https://doi.org/10.1016/j.jafr.2021.100263.
10) Mirković M, Seratlić S, Kilcawley K, Mannion D, Mirković N, Radulović Z. The Sensory Quality and Volatile Profile of Dark Chocolate Enriched with Encapsulated Probiotic Lactobacillus plantarum Bacteria. *Sensors*. 2018;18(8):2570–2570. Available from: https://doi.org/10.3390/s18082570.
11) Amarantini C, Prakasita VC, Sema MT. Preliminary Selection of Potential Probiotic Lactic Acid Bacteria Isolated from Cocoa Beans (Theobroma cacao). *Biosfer: Jurnal Tadris Biologi*. 2021;12(1):1–8. Available from: http://ejournal.radenintan.ac.id/index.php/biosfer/article/view/7128.
12) Oberoi K, Tolun A, Altintas Z, Sharma S. Effect of Alginate-Microencapsulated Hydrogels on the Survival of Lactobacillus rhamnosus under Simulated Gastrointestinal Conditions. *Foods*. 1999;10(9). Available from: https://doi.org/10.3390/foods10091999.
13) Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, et al. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*. 2019;14(4):991–1014. Available from: https://doi.org/10.1038/s41596-018-0119-1.
14) Kazancıgil E, Demirci T, Öztürk-Negiş Hİ, Akın N. Isolation, technological characterization and in vitro probiotic evaluation of Lactococcus strains from traditional Turkish skin bag Tulum cheeses. *Annals of Microbiology*. 2019;69(12):1275–1287. Available from: https://doi.org/10.1007/s13213-019-01512-4.
15) Wu H, Liu Z, Lu P, Barrow C, Dunshea FR, Suleria HAR. Bioaccessibility and bioactivities of phenolic compounds from roasted coffee beans during in vitro digestion and colonic fermentation. *Food Chemistry*. 2022;386:132794. Available from: https://doi.org/10.1016/j.foodchem.2022.132794.
16) Priyadarshana RMA, Daniel CR. Analysis of Antibiotic Resistance of the Probiotic Bacteria Found in Commercial Food Products. *Vaccination Research – Open Journal*. 2019;4(1):30–37. Available from: https://openventio.org/wp-content/uploads/Analysis-of-Antibiotic-Resistance-of-the-Probiotic-Bacteria-Found-in-Commercial-Food-Products-VROJ-4-115.pdf.
17) Pandey KK, Sood SK, Verma SK, Kumar S, Rani S, Ganguli S. Bioutilization of paneer whey waste for production of paneer making powder containing pediocin PA-1 as a biopreservative to enhance shelf life of paneer. *LWT*. 2019;113:108243. Available from: https://doi.org/10.1016/j.lwt.2019.06.010.
18) Ishara S, Gunasena DK. Heat Resistant Moulds in Pasteurized Fruit Syrups. *European Journal of Agriculture and Food Sciences*. 2021;3(1):104–111. Available from: https://doi.org/10.24018/ejfood.2021.3.1.199.
19) c Petronijevi´ c JL, c JPR, c DO, c ZR, c DP, c MP, et al. Viability of probiotic strains Lactobacillus acidophilus NCFM® and Bifidobacterium lactis HN019 and their impact on sensory and rheological properties of milk and dark chocolates during storage for 180 days. *Journal of Functional Foods*. 2015;15:541–550. Available from: https://doi.org/10.1016/j.jff.2015.03.046.
20) Champagne CP, Guertin N, Raymond Y. Strategies to improve the survival of probiotic Lacticaseibacillus rhamnosus R0011 during the production and storage of granola bars. *Canadian Journal of Microbiology*. 2022;68(3):147–156. Available from: https://doi.org/10.1139/cjm-2021-0130.
21) Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA, Kopsahelis N. Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. *Nutrients*. 2019;11(7):1591–1591.
22) Kvakova M, Bertkova I, Stofilova J, Savidge TC. Co-Encapsulated Synbiotics and Immobilized Probiotics in Human Health and Gut Microbiota Modulation. *Foods*. 2021;10(6):1297–1297. Available from: https://doi.org/10.3390/foods10061297.
23) Alinovi M, Mucchetti G, Wiking L, Corredig M. Freezing as a solution to preserve the quality of dairy products: the case of milk, curds and cheese. *Critical Reviews in Food Science and Nutrition*. 2021;61(20):3340–3360. Available from: https://doi.org/10.1080/10408398.2020.1798348.
24) De Rezende LP, Barbosa J, Teixeira P. Analysis of Alternative Shelf Life-Extending Protocols and Their Effect on the Preservation of Seafood Products. *Foods*. 2022;11(8):1100. Available from: https://doi.org/10.3390/foods11081100.

25) Rolfe C, Daryaei H. Intrinsic and Extrinsic Factors Affecting Microbial Growth in Food Systems. *Food Engineering Series*. 2020;p. 3–24. Available from: https://doi.org/10.1007/978-3-030-42660-6_1.
26) Foong YJ, Lee ST, Ramli N, Tan YN, Ayob MK. Incorporation of Potential Probiotic Lactobacillus plantarum Isolated from Fermented Cocoa Beans into Dark Chocolate: Bacterial Viability and Physicochemical Properties Analysis. *Journal of Food Quality*. 2013;36(3):164–171. Available from: https://onlinelibrary.wiley.com/doi/epdf/10.1111/jfq.12028.
27) Settier-Ramírez L, López-Carballo G, Gavara R, Hernández-Muñoz P. Broadening the antimicrobial spectrum of nisin-producing Lactococcus lactis subsp. Lactis to Gram-negative bacteria by means of active packaging. *International Journal of Food Microbiology*. 2021;339:109007. Available from: https://doi.org/10.1016/j.ijfoodmicro.2020.109007.
28) Sharouba A, Mahmoud M. Microbiological Evaluation of Meals Served To Students in the University City Restaurant of Benha University. *Annals of Agricultural Science, Moshtohor*. 2023;61(1):143–150. Available from: https://doi.org/10.21608/ASSJM.2023.190890.1208.