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Article

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Process Optimisation and Quality Evaluation of a Vitamin D_3-Fortified, Monk Fruit–Sweetened Fermented Milk Beverage Containing *Shatavari* and *Ashwagandha*

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The combined incorporation of Ayurvedic botanicals, vitamin D_3 and alternative sweeteners into a fermented milk beverage (FMB) presents technological challenges related to fermentation, texture, phytochemical characteristics and sensory acceptance. This study developed a vitamin D_3-fortified FMB containing Shatavari (*Asparagus racemosus*) and Ashwagandha (*Withania somnifera*) and evaluated the feasibility of completely replacing sucrose with a commercial monk fruit-based bulk sweetener. A central composite rotatable design was used to optimise the concentrations of Shatavari, Ashwagandha and sucrose based on colour and appearance, body and texture, sweetness, flavour and overall acceptability. Sucrose was subsequently replaced with the monk fruit-based sweetener at concentrations ranging from 8.5 to 14.5% (w/v, based on milk volume). The optimised sucrose-sweetened herbal beverage (S-HFMB), monk fruit-sweetened herbal beverage (MF-HFMB) and non-herbal control beverage (C-FMB) were evaluated for quality parameters. Quadratic models adequately described all sensory responses (R^2 = 0.9559–0.9752), with maximum scores observed at intermediate ingredient concentrations. Numerical optimisation identified 0.35% Shatavari, 0.35% Ashwagandha and 12.50% sucrose as optimal, yielding a predicted overall acceptability score of 8.66 and a composite desirability of 0.96. Complete replacement of sucrose with 12.5% monk fruit-based sweetener produced the highest overall acceptability

score (8.94/9). MF-HFMB contained 24.52% total solids, 3.50% protein and 4.10% fat and exhibited greater apparent viscosity than C-FMB (22.10 versus 20.40 mPa·s). Its DPPH radical-scavenging activity was 0.92 mmol Trolox equivalents (TE) L^{-1}, compared with 0.41 mmol TE L^{-1} in C-FMB, while its measured vitamin D_3 concentration was 11.88 µg 100 mL^{-1}. MF-HFMB also exhibited greater mean *in vitro* antibacterial activity against the tested bacteria than C-FMB (19.63 versus 12.27 mm) while maintaining high presumptive lactobacilli and streptococci counts of 8.878 and 9.430 $\log_{10}$ CFU mL^{-1}, respectively. A sensorially acceptable, vitamin D_3-fortified herbal fermented milk beverage can be produced with complete sucrose replacement using a commercial monk fruit-based bulk sweetener. MF-HFMB showed higher DPPH radical-scavenging activity and apparent viscosity than C-FMB, with high presumptive starter-group counts and greater *in vitro* antibacterial activity. These findings support further evaluation under refrigerated-storage and pilot-scale conditions.

Keywords: *Asparagus racemosus*; *Withania somnifera*; fermented milk beverage; monk fruit-based bulk sweetener; vitamin D_3 fortification; functional dairy product

1. INTRODUCTION

A fermented milk beverage (FMB) is increasingly investigated as a delivery matrix for lipophilic vitamins, plant-derived bioactive compounds and reduced-sugar formulations because milk constitutes a multiphase colloidal system containing aqueous, protein-colloidal and dispersed lipid domains (Lavelli and Pellegrino 2021; Rashwan et al. 2023). *Lassi* is a traditional Indian FMB generally produced by stirring or blending *dahi* or yoghurt with water and, depending on the formulation, sugar, salt, fruits, spices or flavouring ingredients (Prasad et al. 2026). During lactic fermentation, acidification towards the isoelectric region of caseins (approximately pH 4.6) decreases electrostatic and steric stabilisation, promotes solubilisation of colloidal calcium phosphate and induces casein-micelle aggregation into a three-dimensional gel network (Lucey, 2002; Juste et al. 2025). In heat-treated fermented-milk

products, denatured whey protein–κ-casein complexes may become incorporated into this network, whereas water and soluble serum constituents occupy its pores; fat globules can behave as active or inactive structural fillers depending on homogenisation, interfacial protein coverage and processing history. This multiphase structure provides potential compartments for ingredients of differing polarity. In particular, the hydrophobicity of vitamin D_3 means that its incorporation, stability and gastrointestinal bioaccessibility are influenced by the lipid fraction, interactions with milk proteins and the delivery system employed (Lavelli and Pellegrino 2021).

Botanical powders may introduce dietary fibre, saponins, polyphenols and insoluble particles that alter serum-binding capacity, protein–polyphenol interactions, gel-network organisation, colour, syneresis and apparent viscosity (Yildirim-Elikoglu and Erdem 2018; Rashwan et al. 2023). Shatavari (*Asparagus racemosus*) extract, for example, has been shown to interact with milk proteins and modify the colour characteristics of milk (Veena et al. 2015). Depending on their concentration, particle characteristics and phytochemical composition, such ingredients may also impart graininess, bitterness, astringency or earthy flavours that reduce sensory acceptance. Similarly, replacing sucrose with a monk fruit-based sweetener can modify fermentation behaviour, rheological properties, sweetness perception, sourness, bitterness, astringency and consumer liking (Ban et al. 2020; Buchilina and Aryana 2021; Chadha et al. 2022). For a commercial 1:1 bulk sweetener, however, these effects cannot be attributed to mogrosides alone because total solids, water activity, osmotic conditions, carbohydrate availability and mouthfeel are also determined by the type and concentration of the carrier or bulking agent. Consequently, simultaneous incorporation of botanical powders, vitamin D_3 and a monk fruit-based bulk sweetener requires formulation optimisation to balance nutrient delivery, fermentation performance, colloidal stability and sensory acceptability.

Vitamin D deficiency remains a widespread nutritional concern because relatively few foods naturally contain substantial quantities of the vitamin, and unfortified yoghurt and other fermented dairy products generally provide only small amounts (Gasparri et al. 2019; Deepika et al. 2025). Fortification with vitamin D_3 (cholecalciferol) therefore represents a practical food-based approach for improving the nutritional value of yoghurt-based fermented milk beverages (Zahedirad et al. 2019; Wong et al. 2025). Owing to its pronounced lipophilicity and limited aqueous solubility, vitamin D_3 may associate with milk-fat globules and dispersed lipid droplets, while casein micelles and other protein-based colloidal structures may facilitate its dispersion and retention within the milk matrix (Lavelli and Pellegrino 2021). The effectiveness of yoghurt as a delivery vehicle is supported by Gasparri et al. (2019), who reported that consumption of vitamin D-fortified yoghurt increased serum 25-hydroxyvitamin D [25(OH)D] by approximately 31 nmol L^{-1} relative to control treatments. Moreover, vitamin D_3-fortified yoghurt and yoghurt drinks likely improve circulating 25(OH)D concentrations (Wong et al. 2025).

The technological success of fortification depends on the physicochemical form and dispersibility of vitamin D_3, the carrier type, stage of addition, mixing and homogenisation conditions, product fat content, storage environment and the light- and oxygen-barrier properties of the packaging material (Kazmi et al. 2007; Zahedirad et al. 2019; Lavelli and Pellegrino 2021). Jafari et al. (2016) demonstrated that both water- and oil-dispersible vitamin D_3 were retained during yoghurt processing and refrigerated storage when the products were packed in opaque containers. In contrast, vitamin D_3 concentrations reduced considerably during storage in translucent containers, demonstrating the importance of protection against light exposure. In botanically fortified fermented milk beverages, additional matrix complexity may arise from suspended plant particles and protein–polyphenol interactions. These

components could potentially modify lipid–water interfacial properties, vitamin D_3 distribution and analytical measurement.

Asparagus racemosus Willd. (Shatavari) and *Withania somnifera* (L.) Dunal (Ashwagandha) are Ayurvedic medicinal plants with emerging relevance as functional food ingredients. Shatavari root contains steroidal saponins, particularly shatavarins, together with polysaccharides, flavonoids and other phenolic constituents, which have been reported to exhibit antioxidant, immunomodulatory, anti-inflammatory and galactagogue activities (Akhtar et al. 2024). Ashwagandha root is characterised principally by withanolides, withanosides and alkaloids. These constituents have exhibited antioxidant, anti-inflammatory and neuroactive properties and potential stress- and anxiety-reducing effects (Bhat et al. 2022; Arumugam et al. 2024). Shatavari extract may also interact with milk proteins and modify the physicochemical properties of milk (Veena et al. 2015). However, phytochemical composition can vary with genotype, growing conditions, plant part, harvest maturity and post-harvest processing. Moreover, direct incorporation of root powders introduces suspended insoluble material and plant-associated flavours that may influence acidification, water retention, rheology, colour and sensory acceptance (Rashwan et al. 2023).

Sweetness is a major determinant of the sensory acceptance of herbal fermented milk beverages because it modulates perceived acidity and can partially mask the bitter, earthy and astringent flavours associated with botanical ingredients. However, concerns regarding the amount of added sucrose in sweetened dairy products have stimulated interest in alternative sweeteners. Monk fruit, obtained from *Siraitia grosvenorii*, contains intensely sweet cucurbitane-type triterpene glycosides, also termed mogrosides, among which mogroside V is the predominant sweetness-contributing compound. Studies in conventional and camel-milk yoghurt demonstrate that monk fruit sweeteners can affect fermentation behaviour, pH, colour, apparent viscosity, and rheological characteristics in addition to sweetness and aftertaste (Ban et al.

2020; Buchilina and Aryana 2021). Moreover, monk fruit-sweetened yoghurt may exhibit persistent bitter, liquorice-like or astringent sensations that negatively influence consumer liking (Chadha et al. 2022).

These effects are strongly dependent on sweetener composition and application level. Purified monk fruit extracts are high-intensity sweeteners and are therefore used at substantially lower concentrations than sucrose. In contrast, commercial sweeteners intended for 1:1 sucrose replacement generally combine monk fruit-derived mogrosides with a bulk carrier, such as erythritol or another carbohydrate or polyol, to approximate the sweetness, mass and handling properties of sucrose. Consequently, the functional behaviour of these formulations is influenced by mogroside concentration as well as the type and proportion of the carrier. Changes in bulk-solids contribution, fermentable-carbohydrate availability, water distribution and sweetness–acidity balance may influence fermentation kinetics, viscosity, mouthfeel and sensory acceptance. Therefore, a commercial 1:1 monk fruit-based sweetener must be evaluated as a complete formulation rather than considered technologically equivalent to purified monk fruit extract.

Although the separate application of Ayurvedic botanicals, vitamin D_3 and monk fruit sweeteners in milk matrices has been reported, their simultaneous incorporation into a single fermented milk beverage remains insufficiently investigated. To the best of our knowledge, the combined addition of Shatavari and Ashwagandha to a vitamin D_3-fortified fermented milk beverage, followed by complete sucrose replacement with a commercial 1:1 monk fruit-based sweetener, has not previously been evaluated. This formulation presents several potentially competing effects: increasing botanical-solids concentrations may enhance phytochemical content but intensify bitterness, astringency and particulate mouthfeel, whereas replacing sucrose with a carrier-containing monk fruit sweetener may alter flavour masking, total solids, water distribution and apparent viscosity. Accordingly, the study was conducted to optimise

the formulation to balance nutritional enrichment, fermentation performance, physical stability and sensory acceptability.

2. MATERIALS AND METHODS

2.1 Raw materials, starter culture and analytical reagents

Mixed cow–buffalo milk was obtained from a local dairy farm in Amreli district, Gujarat, India. A freeze-dried, direct-vat-set thermophilic yoghurt culture (DELVO® YOG FVV-411 DSL; Danisco India Pvt. Ltd., Mumbai, India), containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, was stored at −18 °C until use. Food-grade root powders of Shatavari (*Asparagus racemosus* Willd.) and Ashwagandha (*Withania somnifera* (L.) Dunal) were procured from a certified Ayurvedic botanical supplier. Food-grade crystalline cane sucrose was purchased locally and finely ground before incorporation. A commercial monk fruit-based bulk sweetener formulated as a 1:1 sucrose replacer (Vitasierra Monk Fruit Sweetener Organic 1:1 Sugar Replacer, Aurangabad, Maharashtra, India) was used. Food-grade vitamin D_3 premix (Dhruvcal, Bengaluru, Karnataka, India) was stored in an airtight, light-resistant container according to the manufacturer's recommendations. Vitamin D_3 reference standard, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox were obtained from Sigma-Aldrich, USA. De Man–Rogosa–Sharpe agar and M17 agar were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, Maharashtra, India).

2.2 Experimental design and formulation optimisation

The concentrations of *Shatavari*, *Ashwagandha* and sucrose were optimised using response surface methodology (RSM) with a three-factor, five-level central composite rotatable design generated in Design-Expert® version 13 (Stat-Ease Inc., Minneapolis, MN, USA). The factorial ranges were 0.20–0.50% (w/v) for Shatavari (A), 0.20–0.50% (w/v) for Ashwagandha (B) and 8.0–17.0% (w/v) for sucrose (C), with centre points of 0.35, 0.35 and 12.5%, respectively. Using an axial distance of ±1.682, the complete experimental ranges extended

from approximately 0.098 to 0.602% for each botanical powder and from 4.93 to 20.07% for sucrose. The design comprised 20 randomised experimental runs: eight factorial points, six axial points and six replicated centre points. Vitamin D_3 addition, milk composition, starter-culture inoculation, water addition and processing conditions were maintained constant across all formulations. Colour and appearance, body and texture, sweetness, flavour and overall acceptability, evaluated using a nine-point hedonic scale, were selected as response variables.

2.3 Preparation of vitamin D_3-fortified herbal FMB containing *Asparagus racemosus* and *Withania somnifera*

Each formulation was manufactured as an independent 1-L final-product batch as described in Figure 1. Standardised mixed milk containing 4.5% fat and 8.5% solids-not-fat was filtered, preheated to 40 ± 2 °C and supplemented with the specified quantities of Shatavari, Ashwagandha and sweetener. The ingredients were dispersed by mechanical stirring at 500–700 rpm for 5 min to minimise powder agglomeration. The mixture was heated to 90 ± 1 °C, held for 5 min under continuous gentle agitation and cooled rapidly to 42 ± 1 °C. DELVO® YOG FVV-411 DSL culture was added at 0.02% (w/v; 0.20 g L^{-1}), and the inoculated milk was incubated at 42 ± 1 °C until pH 4.60 ± 0.05 and titratable acidity of 0.72 ± 0.02% lactic acid were attained, which required approximately 6–7 h.

The fermented coagulum was cooled to ≤10 °C within 30 min and stabilised at 7 ± 1 °C for 5–6 h to restrict post-acidification. The coagulum was subsequently disrupted using a laboratory blender at 8000 rpm for 2 min to produce a smooth, lump-free consistency. Pasteurised potable water, cooled to ≤10 °C, was incorporated at 10% (v/v) of the final formulation. Vitamin D_3 was then added under subdued light to provide 6000 IU L^{-1} of finished beverage, equivalent to 150 µg L^{-1} or 600 IU per 100-mL serving. Final blending was performed at 5000 rpm for 60 s, with minimum headspace and aeration to limit oxidative deterioration. The beverage was filled

195 into sanitised opaque 100-mL food-grade cups, sealed immediately with aluminium foil and
196 stored at 7 ± 1 °C.

197 For the sucrose-replacement experiment, the botanical concentrations were fixed at the
198 optimised levels of 0.35% (w/v) Shatavari and 0.35% (w/v) Ashwagandha. Sucrose was
199 completely replaced on a 1:1 mass basis with the commercial monk fruit-based bulk sweetener
200 at 8.5, 10.5, 12.5 or 14.5% (w/v, based on milk volume). The sweetener was incorporated at
201 the same pre-fermentation stage used for sucrose, while milk composition, culture
202 concentration, heat treatment, fermentation endpoint, water addition, vitamin D_3 fortification,
203 blending, packaging and storage conditions were maintained constant.

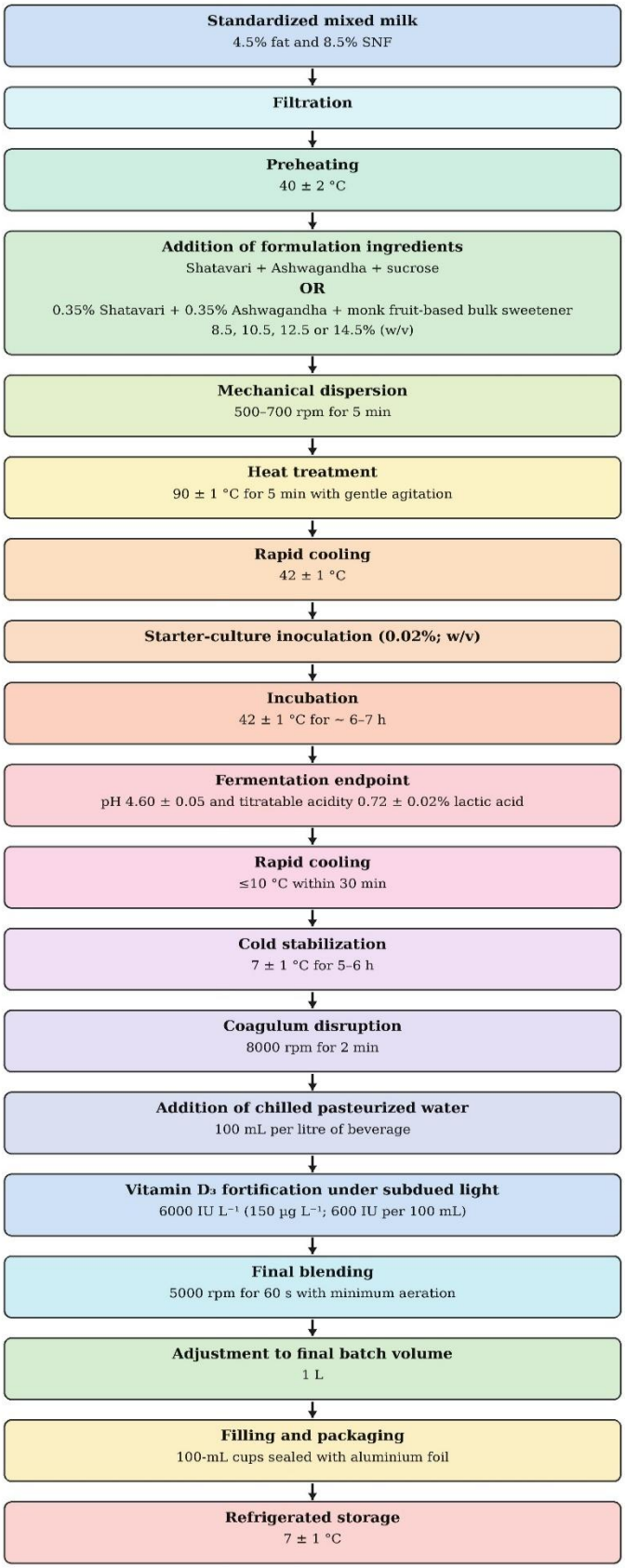

Standardized mixed milk
4.5% fat and 8.5% SNF
Filtration
Preheating
40 ± 2 °C
Addition of formulation ingredients
Shatavari + Ashwagandha + sucrose
OR
0.35% Shatavari + 0.35% Ashwagandha + monk fruit-based bulk sweetener
8.5, 10.5, 12.5 or 14.5% (w/v)
Mechanical dispersion
500–700 rpm for 5 min
Heat treatment
90 ± 1 °C for 5 min with gentle agitation
Rapid cooling
42 ± 1 °C
Starter-culture inoculation (0.02%; w/v)
Incubation
42 ± 1 °C for ~ 6–7 h
Fermentation endpoint
pH 4.60 ± 0.05 and titratable acidity 0.72 ± 0.02% lactic acid
Rapid cooling
≤10 °C within 30 min
Cold stabilization
7 ± 1 °C for 5–6 h
Coagulum disruption
8000 rpm for 2 min
Addition of chilled pasteurized water
100 mL per litre of beverage
Vitamin D₃ fortification under subdued light
6000 IU L⁻¹ (150 µg L⁻¹; 600 IU per 100 mL)
Final blending
5000 rpm for 60 s with minimum aeration
Adjustment to final batch volume
1 L
Filling and packaging
100-mL cups sealed with aluminium foil
Refrigerated storage
7 ± 1 °C

Figure 1 Process flowchart for manufacturing vitamin D_3-fortified FMBs containing *Asparagus racemosus* and *Withania somnifera*, followed by complete replacement of sucrose with a commercial 1:1 monk fruit-based bulk sweetener

2.4 Sensory evaluation of FMBs

Sensory evaluation was performed by 20 trained assessors (23–49 years of age) selected on the basis of sensory acuity, availability, previous experience with fermented dairy products and absence of known allergies or intolerances to the ingredients. Before evaluation, assessors received one week of training on the sensory attributes and use of the nine-point hedonic scale (1 = dislike extremely; 9 = like extremely). Beverages were served at 10 ± 2 °C in 30-mL portions in identical odour-free cups bearing randomised three-digit codes. Samples were presented monadically in a randomised and balanced order under uniform white illumination in a quiet, odour-free sensory laboratory. Drinking water and unsalted crackers were provided for palate cleansing, with a 1-min interval between samples. Colour and appearance, flavour, sweetness, body and texture, and overall acceptability were evaluated. Each formulation, prepared in three independent batches, was assessed during three sessions, with no more than six samples presented per session to minimise fatigue. Assessors were informed that the beverages contained milk, starter culture, Shatavari, Ashwagandha and either sucrose or a monk fruit-based sweetener; written informed consent was obtained, and the protocol was reviewed by the Research Committee of the College of Dairy Science, Kamdhenu University, Amreli, Gujarat, India.

2.5 Compositional and physicochemical analyses

The optimised sucrose- and monk fruit-sweetened fermented milk beverages were analysed for total solids, fat, protein, ash and lactose content following methods described by BIS (1981) and AOAC International (2019). Titratable acidity and pH were also determined using standard methods. Apparent viscosity was measured using a controlled-stress rheometer (MCR 92,

Anton Paar GmbH, Graz, Austria) fitted with a 50-mm parallel-plate geometry. Before measurement, the beverage was gently mixed to ensure homogeneity without incorporating air and equilibrated at 25 ± 1 °C. Approximately 2.0 mL of sample was placed on the lower plate, the measuring gap was set at 1.0 mm, and excess sample was carefully removed. After equilibration for 2 min, apparent viscosity was measured at a constant shear rate of 50 s^{-1} and expressed as mPa·s. A fresh sample was loaded for each measurement, and three measurements were performed for each independently manufactured batch.

2.6 Free fatty acids

Free fatty acids were determined by solvent-extraction titration. A beverage sample (3.0 g) was dispersed in 5.0 mL of distilled water and mixed with 10 mL of isopropanol: petroleum ether:4 N H_2SO_4 (40:10:1, v/v/v). Petroleum ether (6.0 mL) was added, and the sealed tube was maintained at 40 ± 1 °C for 10 min, shaken vigorously for 20 s and allowed to separate for 5–10 min. A 5.0-mL aliquot of the upper phase was titrated with standardised 0.02 N methanolic KOH using two drops of 1% methanolic α-naphtholphthalein. A reagent blank was analysed concurrently. Results were expressed as μequivalents g^{-1} of beverage

2.7 Tyrosine value

Tyrosine value was determined using a modified TCA-soluble Lowry–Folin procedure. A beverage sample (5.0 g) was mixed with 5.0 mL of distilled water and 5.0 mL of 30% (w/v) trichloroacetic acid, vortexed for 1 min, held for 10 min and filtered through Whatman No. 2 paper. The filtrate (0.20 mL) was mixed with 5.0 mL of alkaline copper–tartrate reagent containing 4 g $CuSO_4 \cdot 5H_2O$, 24 g anhydrous Na_2CO_3, 16 g potassium sodium tartrate and 180 g anhydrous Na_2SO_4 L^{-1}. After 10 min at 25 ± 1 °C, 0.5 mL of 0.667 N Folin–Ciocalteu reagent was added. The mixture was incubated for 30 min, diluted to 10 mL and measured at 700 nm using a UV-1900i spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A calibration

curve was prepared using L-tyrosine standards of 0–45 µg mL^{-1}. Results were expressed as µg tyrosine equivalents g^{-1} beverage.

$$\text{Tyrosine Value } (\mu\text{eq g}^{-1}) = \frac{C \times V_e \times DF}{W}$$

where C is the concentration obtained from the calibration curve, V_e is the extraction volume, DF is the dilution factor and W is the sample mass.

2.8 DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined following Brand-Williams et al. (1995), with modifications. The beverage sample was diluted to a final dilution factor of 2.5 by mixing one volume of sample with 1.5 volumes of Milli-Q water and vortexing for 1 min. A 10-mL aliquot was centrifuged at 5500 rpm for 10 min at 4 ± 1 °C, and the recovered supernatant was centrifuged again under identical conditions. The clear supernatant was collected in aluminium-foil-covered tubes, equilibrated to 25 ± 1 °C and analysed immediately. A 2.81-mM DPPH stock solution was prepared by dissolving 27.7 mg of DPPH in 25 mL of methanol in an amber bottle. Immediately before analysis, 2.5 mL of this stock was diluted to 100 mL with methanol to obtain a 70.25-µM working solution. The working solution was adjusted with methanol to an absorbance of 0.70 ± 0.02 at 515 nm. A 5-mM Trolox stock solution was prepared by dissolving 12.5 mg of Trolox in 10 mL of ethanol and diluted to obtain working standards of 100–1000 µM. For the assay, 100 µL of sample supernatant or Trolox standard was mixed with 3.9 mL of freshly prepared DPPH working solution, vortexed for 10 s and incubated at 25 ± 1 °C for 30 min in the dark. After incubation, absorbance was measured at 515 nm using a UV–visible spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan) equipped with matched 1-cm quartz cuvettes. The control contained 100 µL of Milli-Q water and 3.9 mL of DPPH working solution, while the sample blank contained 100 µL of

sample supernatant and 3.9 mL of methanol to correct for sample colour and turbidity. DPPH radical-scavenging activity was calculated as:

$$\text{DPPH inhibition (\%)} = \frac{A_0 - (A_s - A_b)}{A_0} \times 100$$

where A_0 is the absorbance of the DPPH control, A_s is the absorbance of the sample–DPPH reaction and A_b is the absorbance of the sample blank.

2.9 UV-spectrophotometric quantification of vitamin D_3

Vitamin D_3 was extracted and quantified following the method of Rahman et al. (2019), with some modifications. A vitamin D_3 standard solution (100 µg mL^{-1}) was prepared in HPLC-grade methanol and appropriately diluted to obtain working standards of 0.5–3.5 µg mL^{-1}. The absorbance of each working standard was measured at 275 nm using a UV–visible spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan) equipped with matched 1-cm quartz cuvettes, with methanol used for baseline correction. A calibration curve was prepared by plotting absorbance against vitamin D_3 concentration. For sample extraction, 2.0 mL of thoroughly homogenised FMB was mixed with 4.0 mL of methanol:isopropanol (80:20, v/v) and vortexed for 2 min to precipitate proteins and disrupt the emulsion. Vitamin D_3 was extracted successively three times with 4.0 mL of *n*-hexane; after each addition, the mixture was vortexed for 60 s and centrifuged at 4000 rpm for 15 min. The upper organic layers were collected, pooled and evaporated to dryness in a water bath at 60 ± 2 °C under reduced light. The residue was reconstituted in 5.0 mL of methanol and mixed on an orbital shaker at 40 rpm and 25 ± 1 °C for 1 h. The reconstituted extract was clarified through a 0.45-µm PTFE syringe filter, and its absorbance was measured at 275 nm against a blank. An unfortified beverage blank was processed concurrently to correct for absorbance from co-extracted milk and botanical constituents. Vitamin D_3 concentration was calculated from the calibration equation,

corrected for the sample, reconstitution and dilution volumes, and converted to international units using 1 µg cholecalciferol = 40 IU.

2.10 Antimicrobial activity by agar-well diffusion

Antimicrobial activity was evaluated against *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. The strains were activated from glycerol stocks on Mueller–Hinton agar. Each culture was adjusted using sterile 0.85% saline to a 0.5 McFarland standard (~1.5×10^8 CFU mL^{-1}) and used within 15 min. Cell-free supernatant was prepared by centrifuging a beverage sample at 10,000 × *g* for 10 min at 4 °C and filtering it through a sterile 0.22-µm membrane. A second portion was adjusted aseptically to pH 6.5 ± 0.1 with sterile 1 N NaOH to distinguish acidity-mediated inhibition from the activity of other soluble constituents.

A 100-µL volume of the standardised bacterial suspension was spread uniformly over 90-mm Petri plates containing 20–25 mL of Mueller–Hinton agar at a depth of 4.0 ± 0.5 mm and pH 7.2–7.4. Three equidistant wells, each 7 mm in diameter, were cut aseptically and sealed at the base with approximately 10 µL of molten agar to prevent leakage. Each well was loaded with 100 µL of native or pH-neutralised cell-free supernatant. The plates were maintained at 25 ± 1 °C for 30 min to permit diffusion and subsequently incubated in an inverted position at 35 ± 2 °C for 18–24 h under aerobic conditions. Cell-free supernatants from the plain fermented milk beverage, the vitamin D_3-fortified beverage without *Asparagus racemosus* and *Withania somnifera*, and the herbal beverage containing both botanicals but no vitamin D_3 were included as formulation controls. Sterile 0.85% saline and a ciprofloxacin disc (5 µg) served as the negative and positive inhibition controls, respectively.

After incubation, the complete inhibition-zone diameter, including the 7-mm well, was measured in two perpendicular directions using a digital calliper, and the mean was expressed in millimetres. A measured diameter of 7 mm indicated no detectable inhibition.

2.11 Microbiological analysis

An aseptically collected beverage sample (1.0 mL) was transferred to 9.0 mL of sterile phosphate-buffered diluent to obtain a 10^{-1} dilution, followed by serial tenfold dilutions in the same diluent; plating was completed within 15 min. Appropriate dilutions (1.0 mL) were pour-plated in duplicate using media maintained at 45 ± 1 °C. Presumptive lactobacilli were enumerated on de Man–Rogosa–Sharpe agar overlaid with 5–7 mL of the same medium and incubated anaerobically at 37 ± 1 °C for 72 h. Presumptive streptococci were enumerated on M17 agar with a 5–7-mL overlay and incubated aerobically at 37 ± 1 °C for 48 h. Countable plates were enumerated, and the results were expressed as $\log_{10}$ CFU mL^{-1}; when no colonies were observed, results were reported as below the method-specific detection limit.

3. RESULTS AND DISCUSSION

3.1 Response surface modelling of the effects of *Shatavari*, *Ashwagandha* and sucrose on the sensory quality of vitamin D_3-fortified herbal fermented milk beverage

3.1.1 Adequacy of the RSM models and overall sensory-response patterns

The concentrations of Shatavari, Ashwagandha and sucrose affected all sensory attributes of the vitamin D_3-fortified herbal FMB (Table 1). Scores for colour and appearance, body and texture, sweetness, flavour and overall acceptability ranged from 5.2–8.9, 5.5–8.9, 5.1–8.9, 5.4–8.9 and 5.5–8.8, respectively. The six centre-point formulations, containing 0.35% each of Shatavari and Ashwagandha and 12.5% sucrose, yielded mean sensory scores of 8.64–8.72, which closely agreed with the corresponding model intercepts. Conversely, sensory acceptance declined at both the lower (4.93%) and upper (20.07%) axial sucrose levels, indicating that an

intermediate sucrose concentration was required to achieve the most favourable sweetness–acidity balance and overall sensory quality.

The fitted quadratic models accounted for 95.59–97.52% of the variation in the sensory responses, with adjusted R^2 values of 0.92–0.95 and adequate-precision values of 12.93–17.57 (Table 2). Although the individual linear terms were nonsignificant ($p > 0.05$), the significant negative quadratic terms ($p < 0.05$) generated concave response surfaces. The negative Shatavari × Ashwagandha interaction coefficients indicated an antagonistic combined effect, whereby simultaneous increases in both botanical concentrations caused a greater reduction in sensory quality than variation in either botanical alone.

3.1.2 Colour and appearance score

Colour and appearance scores ranged from 5.2 to 8.9 (Table 1), with the highest scores observed near the centre of the experimental design and the lowest at the axial minimum sucrose concentration (4.93%). The quadratic model explained 96.24% of the response variation ($R^2 = 0.9624$; adjusted $R^2 = 0.9286$) and yielded an adequate-precision value of 14.22, indicating a satisfactory signal-to-noise ratio. The nonsignificant linear effects ($p > 0.05$) and significant negative quadratic terms ($p < 0.05$) generated a concave response surface, with maximum visual acceptability at intermediate ingredient concentrations (Figure 2). Sucrose exhibited the strongest curvature ($C^2 = -1.08$). The negative Shatavari × Ashwagandha interaction coefficient ($AB = -0.58$) suggested that simultaneous increases in both botanical powders were particularly detrimental to visual acceptability.

At higher botanical concentrations, coloured particles and insoluble root constituents may have increased opacity and visible speckling and promoted sediment formation, thereby reducing colour uniformity. Comparable concentration-dependent alterations in yoghurt colour and appearance have been reported following the incorporation of fibre-rich plant powders and

other natural functional ingredients (Ahmad et al. 2022; Rashwan et al. 2023). Shatavari extract has also been shown to interact with milk proteins and modify the physicochemical characteristics of milk, supporting the possibility of matrix-dependent visual changes (Veena et al. 2015). Moderate botanical incorporation appeared to permit more uniform dispersion while maintaining the characteristic appearance of the fermented milk beverage.

The fitted quadratic regression equation in coded factors was as follows:

$$Colour\ and\ \text{appearance} = 8.6995 - 0.0316A - 0.0809B + 0.0376C - 0.575AB + 0.075AC + 0.125BC - 0.4743A^2 - 0.5804B^2 - 1.0754C^2$$

3.1.3 Body and texture score

Body and texture scores ranged from 5.5 to 8.9. The maximum score was recorded at the centre-point formulation containing 0.35% Shatavari, 0.35% Ashwagandha and 12.5% sucrose, whereas the minimum occurred at the axial minimum sucrose concentration of 4.93%. The quadratic model accounted for 95.59% of the response variation ($R^2 = 0.9559$; adjusted $R^2 = 0.9163$) and had an adequate-precision value of 12.93. The negative quadratic terms generated a concave response surface, with sucrose producing the strongest curvature ($C^2 = -1.02$). The negative Shatavari × Ashwagandha coefficient ($AB = -0.45$) suggested that increasing both botanicals simultaneously reduced textural acceptability. In contrast, the weakly positive Shatavari × sucrose and Ashwagandha × sucrose coefficients indicated only limited compensation by sucrose (Figure 2).

The favourable texture at intermediate ingredient concentrations may reflect an appropriate balance among the acid-induced milk-protein network, serum-phase viscosity, soluble solids and dispersed botanical particles. The texture of fermented milk is governed principally by the formation and subsequent mechanical disruption of the acid-induced casein network (Wang et

al. 2025). Moderate concentrations of botanical polysaccharides and fibre may promote water immobilisation and increase perceived consistency, as fibre-containing ingredients can modify yoghurt viscosity, rheology and mouthfeel (Dello Staffolo et al. 2004; Mousavi et al. 2019). Sucrose may additionally increase serum-phase solids and perceived body.

At higher combined botanical concentrations, however, insoluble particles may interrupt protein network continuity and increase graininess or particulate roughness. Moreover, plant ingredients can either enhance or disrupt yoghurt structure depending on their composition, physical form and concentration (Ahmad et al. 2022; Rashwan et al. 2023). Insufficient sucrose may produce a comparatively thin sensory body, whereas excessive sucrose may impart an overly dense, sticky or mouth-coating sensation. Thus, controlled botanical incorporation supported acceptable consistency, while excessive incorporation impaired smoothness and structural uniformity.

The fitted quadratic regression equation in coded factors was as follows:

$$\text{Body and Texture} = 8.6393 - 0.0270A - 0.0562B - 0.0163C - 0.4500AB + 0.0500AC + 0.0500BC - 0.4929A^2 - 0.5990B^2 - 1.0233C^2$$

3.1.4 Sweetness score

Sweetness scores ranged from 5.1 to 8.9. The minimum score occurred at 4.93% sucrose, whereas the centre-point formulations yielded scores of 8.5–8.9. Increasing sucrose to the axial maximum of 20.07% reduced the score to 6.1, showing that sensory acceptance depended on attaining an intermediate sweetness level rather than simply increasing sucrose concentration. The quadratic model explained 96.63% of the response variation ($R^2 = 0.9663$; adjusted $R^2 = 0.9359$) and had an adequate-precision value of 15.42. Sucrose produced the strongest curvature ($C^2 = -1.10$), resulting in a distinct sensory optimum near the centre of the design space (Figure 2).

At low sucrose concentrations, sweetness was insufficient to balance fermented acidity and the bitter, earthy or astringent flavours associated with the botanical powders. Sweetness intensity and its balance with acidity and off-flavours are important determinants of yoghurt liking, while both inadequate and excessive sweetness can reduce sensory acceptance (Chadha et al. 2022; Mora and Dando 2021). Conversely, excessive sucrose produced an overly sweet and lingering profile that reduced sensory balance and masked the characteristic fermented-milk and herbal flavours.

The negative Shatavari × Ashwagandha coefficient (AB=−0.40) suggested that simultaneous increases in both powders intensified their combined adverse sensory influence. Similar decreases in sensory acceptance at higher concentrations of herbal or particulate plant ingredients have been reported in fortified yoghurts (Hasneen et al. 2020; Mousavi et al. 2019). The positive Shatavari × sucrose coefficient suggested some potential masking of Shatavari-associated flavour by sucrose, whereas the negligible Ashwagandha × sucrose coefficient suggested little corresponding compensation for Ashwagandha. Nevertheless, formulations containing high concentrations of both sucrose and botanical powders remained less acceptable than the centre-point formulations, indicating that increasing sweetness alone could not compensate fully for botanical off-flavours. This agrees with evidence that sweetness–off-flavour interactions depend on both concentration and the qualitative temporal profile of the sweetening system (Mora et al. 2023).

$$\text{Sweetness} = 8.6851 - 0.0662\text{A} + 0.0293\text{B} + 0.0646\text{C} - 0.4000\text{AB} + 0.1500\text{AC} + 0.0000\text{BC} - 0.6246\text{A}^2 - 0.5362\text{B}^2 - 1.10\text{C}^2$$

3.1.5 Flavour score

Flavour scores ranged from 5.4 to 8.9, with the highest scores obtained near the centre of the design space and the lowest at 4.93% sucrose. Among the sensory responses, the flavour model

provided the highest explanatory power (R^2 = 0.9752; adjusted R^2 = 0.9529) and adequate precision (17.57). Although the linear effects were nonsignificant ($p > 0.05$), Shatavari exhibited the largest negative linear tendency (A=−0.1568). The negative Shatavari × Ashwagandha coefficient (AB = −0.4125) suggested that their simultaneous addition at higher concentrations intensified the combined bitter, earthy, root-like and astringent flavour profile. Concentration-dependent changes in flavour have also been reported when herbs or fibre-rich plant powders were added directly to yoghurt (Hasneen et al. 2020; Mousavi et al. 2019).

The sensory characteristics of the botanical powders may be associated with their chemically diverse constituents. Shatavari roots contain steroidal saponins, polysaccharides, flavonoids and other phenolic compounds (Akhtar et al. 2024), whereas Ashwagandha contains withanolides, withanosides and alkaloids (Bhat et al. 2022). Nevertheless, the contribution of individual compounds to perceived bitterness or astringency was not measured in the present study; therefore, this mechanistic explanation remains inferential.

The positive Shatavari × sucrose and Ashwagandha × sucrose coefficients suggested that sucrose partially moderated the sensory influence of the botanicals. At intermediate concentrations, sucrose balanced herbal flavour and fermented acidity without obscuring the beverage's characteristic flavour profile. At 4.93%, insufficient sweetness accentuated acidic and botanical notes, whereas 20.07% sucrose produced an excessively sweet and unbalanced profile. The pronounced negative quadratic effect of sucrose (C^2=−1.11) therefore reflected its contribution to both sweetness and flavour balance and generated a distinct optimum at an intermediate concentration (Figure 2). Similar sensory studies have demonstrated that sweetness, bitterness, astringency and mouth-coating interact dynamically in determining yoghurt liking (Chadha et al. 2022; Mora et al. 2023).

The fitted quadratic regression equation in coded factors was as follows:

$$\text{Flavour} = 8.7211 - 0.1568A - 0.0489B - 0.0190C - 0.4125AB + 0.1125AC + 0.1125BC - 0.6182A^2 - 0.5475B^2 - 1.1132C^2$$

3.1.6 Overall acceptability score

Overall acceptability scores ranged from 5.5 to 8.8, with three centre-point runs attaining the maximum score. The minimum was recorded at the axial minimum sucrose concentration of 4.93%. The formulation containing 0.50% each of Shatavari and Ashwagandha and 8.0% sucrose obtained a comparatively low score of 5.8, demonstrating the adverse sensory combination of relatively high botanical concentrations and insufficient sweetness. The quadratic model explained 97.46% of the response variation (R^2 = 0.9746; adjusted R^2 = 0.9518) and had an adequate-precision value of 16.86. The negative Shatavari × Ashwagandha coefficient (AB = −0.50) suggested that simultaneous increases in both botanical powders reduced overall sensory quality. All quadratic coefficients were negative, with sucrose producing the greatest curvature (C^2 = −1.03); consequently, the response surface was concave and maximum acceptability occurred near the centre of the design space (Figure 2).

The favourable response at intermediate concentrations reflected an appropriate balance among appearance, texture, sweetness and flavour. Moderate botanical incorporation provided the intended herbal character without excessive colour alteration, bitterness, astringency or particulate roughness. This concentration-dependent response is consistent with studies showing that moderate incorporation of plant ingredients can improve the functionality of yoghurt, whereas excessive addition may adversely affect colour, smoothness, flavour and overall acceptance (Ahmad et al. 2022; Hasneen et al. 2020; Mousavi et al. 2019; Rashwan et al. 2023).

Simultaneously, approximately 12.5% sucrose balanced fermented acidity and botanical flavour while contributing sufficient soluble solids to produce an acceptable body. Lower sucrose concentrations resulted in inadequate sweetness and body, whereas higher

concentrations produced excessive sweetness and mouth-coating, sensory effects consistent with the concentration-dependent role of sweeteners in yoghurt perception (Chadha et al. 2022; Mora and Dando 2021). The convergence of the sensory-response maxima around 0.35% Shatavari, 0.35% Ashwagandha and 12.5% sucrose therefore identified this region as the most suitable basis for numerical optimisation.

The fitted quadratic regression equation in coded factors was as follows:

$$Overall\ acceptability = 8.6641 + 0.0047A - 0.0416B - 0.0240C - 0.5000AB + 0.1250AC + 0.0250BC - 0.6081A^2 - 0.5550B^2 - 1.032C^2$$

Table 1 Central composite design and sensory responses of vitamin D₃-fortified herbal FMB

Run	*Shatavari* (% w/v)	*Ashwagandha* (% w/v)	Sucrose (% w/v)	Colour and appearance	Body and texture	Sweetness	Flavour	Overall acceptability
1	0.350	0.350	12.50	8.6	8.6	8.8	8.9	8.8
2	0.500	0.200	17.00	6.8	6.6	6.6	6.4	6.9
3	0.350	0.350	4.93	5.2	5.5	5.1	5.4	5.5
4	0.350	0.350	12.50	8.7	8.6	8.8	8.8	8.8
5	0.200	0.500	17.00	6.9	6.5	6.5	6.6	6.5
6	0.350	0.098	12.50	7.2	7.1	7.2	7.3	7.1
7	0.350	0.602	12.50	6.9	7.0	7.2	7.2	7.0
8	0.350	0.350	12.50	8.8	8.4	8.5	8.8	8.5
9	0.200	0.200	17.00	6.0	6.0	5.9	6.0	5.9
10	0.602	0.350	12.50	7.4	7.3	6.8	6.8	6.8
11	0.350	0.350	12.50	8.4	8.8	8.9	8.6	8.8
12	0.350	0.350	20.07	6.1	6.2	6.1	5.9	5.9
13	0.200	0.500	8.00	7.4	7.2	7.3	7.2	7.3
14	0.350	0.350	12.50	8.9	8.9	8.5	8.5	8.5
15	0.350	0.350	12.50	8.8	8.5	8.6	8.7	8.6
16	0.098	0.350	12.50	7.3	7.4	7.1	7.3	7.0
17	0.500	0.500	17.00	6.1	6.0	6.2	6.0	6.2
18	0.200	0.200	8.00	6.3	6.2	6.1	6.4	6.1
19	0.500	0.500	8.00	5.6	5.8	5.8	5.5	5.8
20	0.500	0.200	8.00	7.5	7.3	6.8	7.0	7.3

Values represent mean sensory scores on a nine-point hedonic scale, where 1 = dislike extremely and 9 = like extremely. The axial levels were reported to three decimal places to distinguish them from the factorial levels. Vitamin D₃ concentration and processing conditions were maintained constant across formulations.

Table 2 Regression coefficients and model-fit statistics for sensory responses of vitamin D₃-fortified fermented milk beverage

Model term	Colour and appearance	Body and texture	Sweetness	Flavour	Overall acceptability
Intercept	8.7000	8.6400	8.6900	8.7200	8.6600
A	−0.0316	−0.0270	−0.0662	−0.1568	0.0047
B	−0.0809	−0.0562	0.0293	−0.0489	−0.0416
C	0.0376	−0.0163	0.0646	−0.0190	−0.0240
AB	−0.5750	−0.4500	−0.4000	−0.4125	−0.5000

AC	0.0750	0.0500	0.1500	0.1125	0.1250
BC	0.1250	0.0500	0.0000	0.1125	0.0250
A^2	−0.4743	−0.4929	−0.6246	−0.6182	−0.6081
B^2	−0.5804	−0.5990	−0.5362	−0.5475	−0.5550
C^2	−1.0800	−1.0200	−1.1000	−1.1100	−1.0300
R^2	0.9624	0.9559	0.9663	0.9752	0.9746
Adjusted R^2	0.9286	0.9163	0.9359	0.9529	0.9518
Adequate precision	14.2214	12.9260	15.4220	17.5720	16.8647

A = *Shatavari* concentration; B = *Ashwagandha* concentration; and C = sucrose concentration. Regression coefficients correspond to coded factor levels. AB, AC and BC represent two-factor interactions, while A^2, B^2 and C^2 represent quadratic effects. Adequate-precision values greater than 4 indicate an acceptable signal relative to model noise.

3.2 RSM-based numerical optimisation of the herbal FMB formulation

Overall acceptability was selected as the optimisation response because it integrates the collective sensory perception of colour and appearance, body and texture, sweetness and flavour. Numerical optimisation was conducted using the desirability-function approach. Shatavari, Ashwagandha and sucrose concentrations were constrained within their factorial ranges, while overall acceptability was set to maximise with an importance level of 3 (Table 3).

Table 3 Constraints used for numerical optimisation of the vitamin D_3-fortified herbal FMB

Variable or response	Goal	Lower limit	Upper limit	Importance
***Shatavari* (% w/v)**	Within range	0.20	0.50	-
***Ashwagandha* (% w/v)**	Within range	0.20	0.50	-
Sucrose (% w/v)	Within range	8.00	17.00	-
Overall acceptability	Maximise	5.50	8.80	3

The numerical solution identified an optimum formulation containing 0.35% Shatavari, 0.35% Ashwagandha and 12.5% sucrose, with a predicted overall acceptability score of approximately 8.66 and an overall desirability of 0.96. The optimum was located near the centre of the experimental domain, consistent with the significant negative quadratic terms and the concave response surfaces observed for the sensory responses. The high desirability indicated that the proposed formulation closely satisfied the specified factor constraints and maximisation objective.

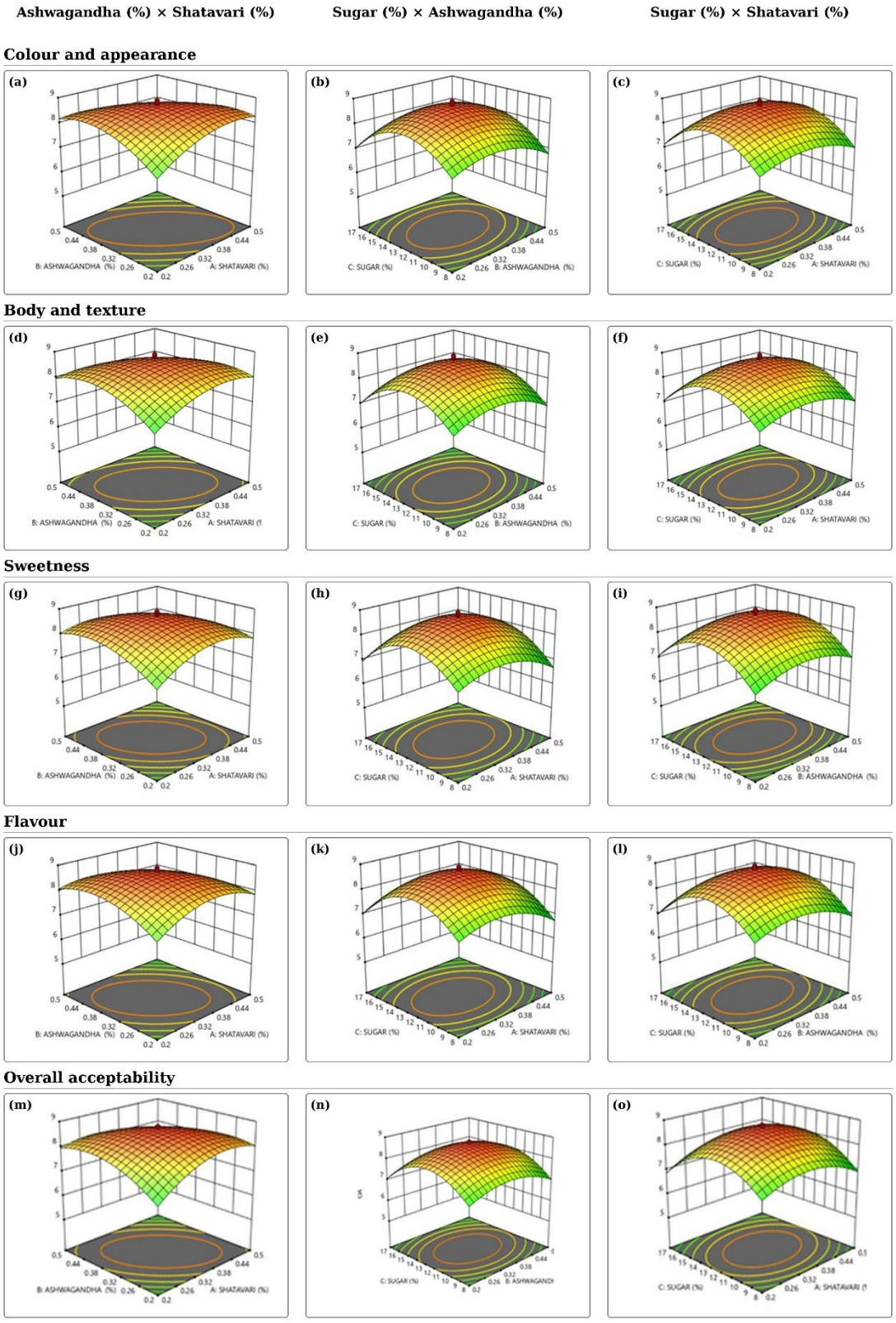


Figure 2 Three-dimensional response-surface and corresponding contour plots showing the interactive effects of Ashwagandha, Shatavari and sucrose concentrations on the sensory attributes of the vitamin D_3-fortified herbal FMB: (a–c) colour and appearance, (d–f) body and texture, (g–i) sweetness, (j–l) flavour and (m–o) overall acceptability. Columns represent the interactions between Ashwagandha and Shatavari, sucrose and Ashwagandha, and sucrose and Shatavari, respectively.

3.3 Optimisation of the commercial monk fruit-based sweetener in vitamin D_3-fortified herbal FMBs

The concentration of the monk fruit-based sweetener powder significantly influenced the sensory profile of the vitamin D_3-fortified herbal FMB (Table 4). Overall acceptability increased from 7.55 at 8.5% to a maximum of 8.94 at 12.5%, followed by a significant decrease to 8.51 at 14.5% ($p < 0.05$). This nonlinear response identified 12.5% (w/v, based on milk volume) as the optimum concentration, providing the most favourable balance among sweetness, flavour, colour and appearance, and body and texture. Similar concentration-dependent effects of monk fruit sweeteners on the sensory and physicochemical properties of fermented dairy products have been reported previously (Ban et al. 2020; Buchilina and Aryana 2021).

3.3.1 Colour and appearance

Colour and appearance scores increased from 8.06 at 8.5% to 8.55 at 10.5% ($p < 0.05$), whereas no significant differences were detected among the 10.5–14.5% treatments ($p > 0.05$). The comparatively low score at 8.5% may reflect reduced visual body or less uniform suspension of botanical particles at the lower concentration of sweetener solids. Buchilina and Aryana (2021) similarly demonstrated that monk fruit sweetener concentration can affect the instrumental colour of fermented camel-milk yoghurt. Beyond 10.5%, however, appearance remained stable, suggesting that product colour within this concentration range was influenced primarily by the milk matrix and dispersed Shatavari and Ashwagandha particles instead of further increases in sweetener concentration.

3.3.2 Body and texture

Body and texture scores increased progressively from 7.59 at 8.5% to 8.94 at 14.5%, with each concentration producing a significantly different response ($p < 0.05$). Because the commercial sweetener was formulated as a 1:1 bulk replacement for sucrose, increasing its concentration

559 likely increased the contribution of its carrier (i.e., allulose) to total solids and modified serum-
560 phase viscosity, mouth-coating and perceived consistency.

561 Monk fruit sweeteners have previously been shown to modify fermentation behaviour,
562 viscosity, rheology and microstructure in conventional and camel-milk yoghurts (Ban et al.
563 2020; Buchilina and Aryana 2021). The low body-and-texture score at 8.5% suggested that this
564 concentration provided insufficient solids or consistency for this type of beverage. Although
565 14.5% produced the highest body-and-texture score, this textural advantage did not maximise
566 overall acceptability because the corresponding sweetness was less preferred. This result
567 demonstrates that perceived thickness alone did not determine overall acceptability; sweetness,
568 flavour and mouthfeel must remain sensorially balanced (Mora and Dando 2021; Wang et al.
569 2025).

570 **3.3.3 Sweetness**

571 Sweetness acceptability exhibited a distinct optimum, increasing from 7.55 at 8.5% to 8.94 at
572 12.5% and subsequently decreasing to 8.59 at 14.5% ($p < 0.05$). At the lower concentrations,
573 sweetness was probably insufficient to balance fermented acidity and the bitter, earthy and
574 astringent flavours associated with the botanical powders. At 12.5%, sweetness effectively
575 moderated such changes without influencing the characteristic fermented-milk profile.

576 The reduction at 14.5% indicated that additional sweetener produced an excessively intense,
577 persistent or otherwise unbalanced sweetness profile. Mogrosides, particularly mogroside V,
578 are high-intensity sweet compounds whose sensory characteristics differ from those of sucrose
579 (Pawar et al. 2013; Mora and Dando 2021). Sensory studies have demonstrated that monk fruit-
580 sweetened yoghurt may exhibit persistent sweetness together with bitter, astringent or
581 liquorice-like sensations, which can reduce liking as consumption progresses (Chadha et al.
582 2022).

3.3.4 Flavour

Flavour scores increased significantly from 8.53 at 8.5% to 8.88 at 10.5% ($p < 0.05$), whereas the scores recorded at 10.5–14.5% were statistically comparable ($p > 0.05$). This response suggested that a minimum sweetener concentration was required to balance fermented acidity and the flavours contributed by Shatavari and Ashwagandha. Increasing the concentration beyond 10.5% provided no additional flavour advantage but did not significantly reduce flavour acceptance.

Natural sweeteners differ considerably from sucrose in sweetness onset, persistence and accompanying bitter, metallic, liquorice-like or astringent sensations, and these effects vary with concentration and food matrix (Mora et al. 2023; Mora and Dando 2021). Within the investigated concentration range, however, the commercial sweetener did not produce an off-flavour sufficiently intense to reduce the flavour score significantly.

3.3.5 Selection of the optimum concentration

The results demonstrate that formulation selection should consider the integrated sensory profile rather than maximisation of an individual attribute. Although 14.5% produced the highest body-and-texture score, its lower sweetness acceptance reduced overall liking. Conversely, 10.5% provided favourable colour and flavour but comparatively lower sweetness and textural scores. The 12.5% treatment combined the highest sweetness and overall-acceptability scores with highly acceptable colour, texture and flavour. Accordingly, 12.5% (w/v, based on milk volume) commercial monk fruit-based sweetener powder was selected for the optimised vitamin D_3-fortified herbal fermented milk beverage.

Table 4 Effect of monk fruit-based sweetener concentration on the sensory attributes of herbal FMB

Monk fruit-based sweetener (% w/v, milk-volume basis)*	Colour and appearance	Body and texture	Sweetness	Flavour	Overall acceptability
8.5	$8.06^{b} \pm 0.057$	$7.59^{d} \pm 0.066$	$7.55^{d} \pm 0.057$	$8.53^{b} \pm 0.047$	$7.55^{c} \pm 0.050$
10.5	$8.55^{a} \pm 0.050$	$8.12^{c} \pm 0.054$	$8.03^{c} \pm 0.050$	$8.88^{a} \pm 0.054$	$8.53^{b} \pm 0.057$
12.5	$8.48^{a} \pm 0.071$	$8.79^{b} \pm 0.066$	$8.94^{a} \pm 0.071$	$8.85^{a} \pm 0.057$	$8.94^{a} \pm 0.054$
14.5	$8.49^{a} \pm 0.066$	$8.94^{a} \pm 0.071$	$8.59^{b} \pm 0.066$	$8.87^{a} \pm 0.047$	$8.51^{b} \pm 0.066$

Values are presented as mean ± SD on a nine-point hedonic scale. Within each sensory attribute, means bearing different lowercase superscripts differ significantly ($p < 0.05$); *Sweetener concentration is expressed as % (w/v, based on milk volume).

3.4 Physicochemical, biochemical and functional characteristics of vitamin D_3-fortified herbal fermented milk beverages and the control fermented milk beverage

3.4.1 Composition and physicochemical characteristics of vitamin D_3-fortified herbal FMBs

Formulation significantly affected the proximate composition of the FMBs (Table 5). Total solids decreased from 25.11% in S-HFMB to 24.52% in MF-HFMB and 24.01% in C-FMB, with a corresponding inverse trend in moisture content ($p < 0.05$). The higher total-solids content of S-HFMB likely reflected the combined contribution of sucrose and the Shatavari and Ashwagandha powders. Incorporation of dried plant ingredients into a fermented milk matrix can increase total solids, carbohydrates, protein and ash, although the magnitude of these changes depends on the composition and concentration of the added material (Rashwan et al. 2023).

Monk fruit extract is an intense sweetener and therefore contributes little bulk when used alone at its effective concentration (Pawar et al. 2013). However, the commercial sweetener used in MF-HFMB contained allulose as a sugar-like bulking ingredient. Allulose is classified as a

low-calorie bulk sweetener and possesses physicochemical properties that permit its use as a partial replacement for the mass and functionality of sucrose (Li et al. 2024). Consequently, the allulose-containing sweetener and botanical powders likely accounted for the higher total-solids content of MF-HFMB relative to C-FMB. Carbohydrate content followed a similar pattern: S-HFMB contained 16.92%, significantly more than MF-HFMB (16.25%) and C-FMB (16.15%), which were statistically comparable. The higher value in S-HFMB was mainly attributable to added sucrose.

Fat content remained statistically comparable among the formulations (4.00–4.10%; $p > 0.05$), indicating that neither botanical incorporation nor replacement of sucrose with the monk fruit–allulose sweetener produced a detectable change in the fat content of the milk base. Protein concentrations were higher in S-HFMB and MF-HFMB than in C-FMB, whereas the two herbal formulations were statistically comparable. This modest increase may partly reflect the contribution of protein and other nitrogen-containing constituents from the root powders (Kohli et al. 2023).

Ash content was also higher in S-HFMB than in C-FMB, while MF-HFMB exhibited an intermediate value that did not differ significantly from either formulation. This directional pattern is consistent with the possible contribution of mineral constituents from the botanical powders and other dry ingredients (Rashwan et al. 2023).

Final pH values were statistically comparable among S-HFMB, MF-HFMB and C-FMB, ranging from 4.60 to 4.63 ($p > 0.05$). These values correspond to the conventional acidification endpoint of yoghurt-type products, near the isoelectric region of casein at which extensive micellar aggregation and gel formation occur (Peng et al. 2009). The results indicate that, under the processing conditions employed, incorporation of Shatavari and Ashwagandha powders and replacement of sucrose with the monk fruit–allulose sweetener did not produce a detectable change in the final pH endpoint.

3.4.2 Fermentation time to endpoint of vitamin D_3-fortified herbal FMBs

Both herbal formulations required 7.0 h to reach the defined fermentation endpoint, significantly longer than the 4.5 h required for C-FMB ($p < 0.05$). The identical fermentation times of S-HFMB and MF-HFMB, despite their different sweetener types, are consistent with the delay being associated with their common Shatavari and Ashwagandha components.

Root-derived phenolics, saponins, polysaccharides and insoluble particles possibly have altered starter-culture activity and acid-induced casein aggregation. Plant polysaccharides and suspended particles may also modify water distribution, protein–particle interactions and the continuity of the developing casein network, thereby changing the time required to reach a setting endpoint (Peng et al. 2009; Rashwan et al. 2023).

Although monk fruit extract has been reported to influence fermentation, pH and rheological development in yoghurt (Ban et al. 2020; Buchilina and Aryana 2021), the absence of a setting-time difference between S-HFMB and MF-HFMB suggests that sweetener replacement was not the primary determinant of the delay under the conditions of the present study.

3.4.3 Apparent viscosity of vitamin D_3-fortified herbal FMBs

Apparent viscosity differed significantly among the fermented milk beverages ($p < 0.05$), decreasing in the order S-HFMB (24.60 ± 0.39 mPa·s) > MF-HFMB (22.10 ± 0.53 mPa·s) > C-FMB (20.40 ± 0.26 mPa·s). Relative to C-FMB, apparent viscosity was 20.6% higher in S-HFMB and 8.3% higher in MF-HFMB. The higher value of S-HFMB than that of the sucrose-sweetened non-herbal control was associated with botanical incorporation and greater resistance of the fermented milk matrix to flow.

This increase may be associated with the polysaccharides, dietary fibre and finely dispersed insoluble particles supplied by the botanical powders. Hydration of these constituents could increase the effective dispersed-phase volume, immobilise serum water and enhance frictional

673 and physical interactions among botanical particles, casein aggregates and the continuous
674 aqueous phase. Plant-derived polysaccharides and fibre may consequently modify the apparent
675 viscosity in a fermented milk matrix (Ba et al. 2025; Rashwan et al. 2023).

676 S-HFMB exhibited 11.3% greater apparent viscosity than MF-HFMB despite both
677 formulations containing the same botanical blend. This difference was therefore associated
678 more closely with the types of sweetener and resulting matrix composition than with botanical
679 concentration. S-HFMB also contained more total solids than MF-HFMB (25.11 versus
680 24.52%), potentially increasing the concentration of dissolved and dispersed constituents and
681 reducing the proportion of freely mobile serum water.

682 The commercial monk fruit sweetener used in MF-HFMB contained allulose as its principal
683 bulking ingredient. Allulose and sucrose differ in molecular structure, hydration behaviour and
684 interactions with water and food biopolymers; replacing sucrose with allulose can therefore
685 modify viscosity in a matrix-dependent manner (Li et al. 2024; Poçan and Çıkrıkcı Erünsal
686 2024). Consequently, the lower apparent viscosity of MF-HFMB probably reflected the
687 combined effects of total-solids concentration, sweetener-associated hydration and interactions
688 among the serum phase, casein aggregates and botanical particles.

689 **3.4.4 Free Fatty acids of vitamin D_3-fortified herbal FMBs**

690 Free fatty acid concentrations differed significantly among the formulations and decreased in
691 the order C-FMB (0.58 μequiv g^{-1}) > MF-HFMB (0.53 μequiv g^{-1}) > S-HFMB (0.49 μequiv
692 g^{-1}; $p < 0.05$). Free fatty acids in fermented dairy products formed by enzymatic hydrolysis of
693 milk triacylglycerols, starter culture activity, processing conditions and fermentation duration
694 (Bao et al. 2016; Chen et al. 2017). The lower concentrations in the herbal formulations are
695 therefore consistent with reduced fatty-acid liberation or availability during fermentation.

Phenolics, saponins, polysaccharides and other constituents supplied by Shatavari and Ashwagandha might have modified microbial lipase or esterase activity, substrate accessibility or the organisation of the milk-fat-globule interface. This mechanism may be plausible because the composition and structural characteristics of the interfacial layer surrounding milk-fat globules regulate the accessibility of triacylglycerols to lipolytic enzymes (Singh and Gallier 2017). Protein–polyphenol association, adsorption of botanical constituents at lipid interfaces and physical obstruction by suspended particles could consequently have influenced the release of free fatty acids.

3.4.5 Tyrosine value of vitamin D_3-fortified herbal FMBs

The tyrosine value differed modestly among the formulations, decreasing from 0.090 mg per 5 mL in C-FMB to 0.080 mg per 5 mL in MF-HFMB and 0.070 mg per 5 mL in S-HFMB. S-HFMB differed significantly from C-FMB, whereas MF-HFMB was statistically intermediate and did not differ from either formulation.

Several matrix-dependent mechanisms could account for this result. The proteolytic system of lactic acid bacteria comprises cell-envelope proteinases that initially hydrolyse caseins, peptide-transport systems and intracellular peptidases that subsequently release smaller peptides and amino acids (Kieliszek et al. 2021). Phenolics, saponins and other constituents of Shatavari and Ashwagandha may have influenced starter-culture proteolytic activity or reduced the accessibility of caseins to such enzymes. Moreover, milk proteins can form non-covalent or covalent complexes with plant polyphenols, potentially changing protein solubility, aggregation and susceptibility to enzymatic hydrolysis (Mao et al. 2024; Tosif et al. 2021).

3.4.6 DPPH radical-scavenging activity of vitamin D_3-fortified herbal FMBs

DPPH radical-scavenging capacity increased substantially following botanical incorporation, reaching 0.92 mmol Trolox equivalents (TE) L^{-1} in MF-HFMB and 0.83 mmol TE L^{-1} in S-

HFMB, compared with 0.41 mmol TE L^{-1} in C-FMB ($p < 0.05$). Relative to C-FMB, the values were approximately 124% higher in MF-HFMB and 102% higher in S-HFMB. The higher values in the herbal formulations are consistent with a contribution from electron- or hydrogen-donating constituents introduced by the botanical powders. Shatavari roots contain steroidal saponins, polyphenols, flavonoids and polysaccharides (Kohli et al. 2023), whereas Ashwagandha preparations contain phenolic acids, flavonoids, withanolides and other compounds exhibiting *in vitro* radical-scavenging activity (Polumackanycz et al. 2023).

MF-HFMB exhibited approximately 10.8% greater DPPH radical-scavenging activity than S-HFMB despite equivalent botanical incorporation, suggesting an additional effect associated with the sweetener. Monk fruit extract has previously increased DPPH-scavenging capacity when incorporated into yoghurt, demonstrating that monk fruit-derived constituents may contribute to the antioxidant activity in a fermented dairy system (Ban et al. 2020).

3.4.7 Vitamin D_3 content of fortified herbal FMBs

Vitamin D_3 concentrations in S-HFMB and MF-HFMB were statistically comparable at 12.00 and 11.88 µg 100 mL^{-1}, respectively ($p > 0.05$), whereas vitamin D_3 was below the method detection limit in C-FMB. Both fortified beverages contained significantly more vitamin D_3 than the control ($p < 0.05$). The close agreement between S-HFMB and MF-HFMB indicates that replacing sucrose with the monk fruit/allulose sweetener powder did not affect the vitamin D_3 concentration determined by the analytical method under the specified conditions.

Relative to the nominal fortification target of 15 µg 100 mL^{-1}, the measured concentrations represented apparent retentions of 80.0% in S-HFMB and 79.2% in MF-HFMB. Previous studies have demonstrated that vitamin D_3 can be incorporated effectively into yoghurt, although measured retention depends on the vitamin preparation, matrix composition,

743 extraction procedure, packaging and storage conditions (Jafari et al. 2016; Kazmi et al. 2007;
744 Maurya et al. 2023).

745 Using the accepted conversion of 1 µg vitamin D = 40 IU, a 100-mL portion contained 480 IU
746 in S-HFMB and 475.2 IU in MF-HFMB (NIH Office of Dietary Supplements, 2025). These
747 quantities corresponded to 80.0% and 79.2%, respectively, of the ICMR–NIN adult RDA
748 reference value of 600 IU day^{-1} (ICMR–NIN, 2020). Thus, a 100-mL portion of either fortified
749 beverage contained a substantial and comparable proportion of the adult RDA requirement
750 value.

751 **Table 5 Physicochemical characteristics of vitamin D_3-fortified herbal fermented milk**
752 **beverages and the control fermented milk beverage**

Parameter	S-HFMB	MF-HFMB	C-FMB
Moisture (%)	74.89 ± 0.08^{c}	75.48 ± 0.08^{b}	75.99 ± 0.08^{a}
Total solids (%)	25.11 ± 0.08^{a}	24.52 ± 0.08^{b}	24.01 ± 0.08^{c}
Fat (%)	4.10 ± 0.05^{a}	4.10 ± 0.05^{a}	4.00 ± 0.05^{a}
Protein (%)	3.40 ± 0.05^{a}	3.50 ± 0.05^{a}	3.20 ± 0.05^{b}
Carbohydrate (%)	16.92 ± 0.08^{a}	16.25 ± 0.08^{b}	16.15 ± 0.08^{b}
Ash (%)	0.69 ± 0.01^{a}	0.67 ± 0.01^{ab}	0.66 ± 0.01^{b}
Titratable acidity (% lactic acid)	0.72 ± 0.01^{a}	0.72 ± 0.01^{a}	0.72 ± 0.01^{a}
pH	4.60 ± 0.02^{a}	4.60 ± 0.02^{a}	4.63 ± 0.02^{a}
Fermentation time to endpoint (h)	7.00 ± 0.10^{a}	7.00 ± 0.10^{a}	4.50 ± 0.10^{b}
Apparent viscosity at 50 s^{-1} (mPa·s)	24.6 ± 0.391^{a}	22.1 ± 0.529^{b}	20.4 ± 0.258^{c}
Free fatty acids (µequiv g^{-1})	0.49 ± 0.01^{c}	0.53 ± 0.01^{b}	0.58 ± 0.01^{a}
Tyrosine value (mg 5 mL^{-1})	0.070 ± 0.005^{b}	0.080 ± 0.005^{ab}	0.090 ± 0.005^{a}

DPPH radical-scavenging activity (mmol TE L^{-1})	0.83 ± 0.02^{b}	0.92 ± 0.02^{a}	0.41 ± 0.02^{c}
Vitamin D_3 (µg 100 mL^{-1})	12.00 ± 0.12^{a}	11.88 ± 0.10^{a}	ND

Values are mean ± SD (n = 3). Within a row, means bearing different lowercase superscripts differ significantly according to Tukey's HSD test ($p < 0.05$). S-HFMB, sucrose-sweetened herbal FMB; MF-HFMB, monk fruit-sweetened herbal FMB; C-FMB, control FMB; TE, Trolox equivalents; ND, not detected

3.5 Antibacterial activity of vitamin D_3-fortified herbal FMBs

Cell-free supernatants from all FMBs inhibited the four test organisms, although the magnitude of inhibition depended significantly on both formulation and bacterial species (Table 6; $p < 0.05$). Against *Staphylococcus aureus*, MF-HFMB and S-HFMB produced comparable inhibition zones of 23.23 and 22.98 mm, respectively, and both were significantly more inhibitory than C-FMB (13.27 mm). The herbal formulations showed inhibition-zone diameters against *S. aureus* that were approximately 73–75% larger than that of C-FMB. Its comparatively high susceptibility may partly reflect the absence of the outer-membrane permeability barrier characteristic of Gram-negative bacteria, allowing diffusible inhibitory compounds more direct access to the cytoplasmic membrane and other cellular targets (Maher and Hassan 2023).

Escherichia coli was the least susceptible test organism, with inhibition-zone diameters of 15.87, 15.57 and 9.75 mm for MF-HFMB, S-HFMB and C-FMB, respectively. Its comparatively lower susceptibility may partly be explained by the Gram-negative cell envelope, in which the lipopolysaccharide-rich outer membrane restricts the penetration of many hydrophobic and amphiphilic compounds, while porin selectivity limits the entry of other solutes. This permeability barrier acts synergistically with multidrug-efflux systems to reduce intracellular accumulation of inhibitory constituents, including certain botanical phytochemicals and fermentation-derived metabolites (Maher and Hassan 2023).

For *Salmonella enterica subsp. enterica serovar Typhimurium*, inhibition-zone diameter decreased significantly from 21.53 mm in MF-HFMB to 20.65 mm in S-HFMB and 14.96 mm in C-FMB. Despite the permeability barrier imposed by its Gram-negative outer membrane, lactic acid can disrupt the outer membrane and enhance the penetration of other inhibitory compounds (Alakomi et al. 2000). The relatively large inhibition zones may therefore reflect the combined effects of acidity and other low-molecular-weight, readily diffusible metabolites or botanical constituents.

Inhibition of *Enterococcus faecalis* followed the same formulation order, with zones of 17.87, 17.15 and 11.08 mm for MF-HFMB, S-HFMB and C-FMB, respectively. Its intermediate susceptibility, despite its Gram-positive envelope, may reflect the substantial stress-tolerance and membrane-homeostasis mechanisms characteristic of enterococci (Gaca and Lemos 2019).

The inhibition produced by C-FMB indicated baseline antibacterial activity in the control formulation. Lactic acid bacteria can produce organic acids, hydrogen peroxide, diacetyl and bacteriocin-like compounds, depending on the strain and fermentation conditions (Mani-López et al. 2022). Undissociated organic acids can enter bacterial cells, and lower intracellular pH as well as lactic acid may also permeabilise the Gram-negative outer membrane, facilitating the entry of other inhibitors (Alakomi et al. 2000).

The greater inhibition observed for S-HFMB and MF-HFMB than for C-FMB may be associated, at least partly, with botanical incorporation. Root extracts of *Asparagus racemosus* and *Withania somnifera* have demonstrated *in vitro* antibacterial activity against test organisms (Mandal et al. 2000; Kumari and Gupta 2015).

Table 6 *In vitro* antibacterial activity of cell-free supernatants from vitamin D_3-fortified herbal FMBs

Formulation	*S. aureus*	*E. coli*	*S. enterica serovar Typhimurium*	*E. faecalis*

S-HFMB	22.98 ± 0.20^{aA}	15.57 ± 0.22^{bD}	20.65 ± 0.24^{bB}	17.15 ± 0.24^{bc}
MF-HFMB	23.23 ± 0.18^{aA}	15.87 ± 0.21^{aD}	21.53 ± 0.17^{aB}	17.87 ± 0.22^{ac}
C-FMB	13.27 ± 0.16^{bB}	9.75 ± 0.18^{cD}	14.96 ± 0.22^{cA}	11.08 ± 0.20^{cc}

Values are inhibition-zone diameters (mm), expressed as mean ± SD (n = 3 independent determinations). Within each test-organism column, means bearing different lowercase superscripts differ significantly among formulations ($p < 0.05$). Within each formulation row, means bearing different uppercase superscripts differ significantly among test organisms ($p < 0.05$). S-HFMB, sucrose-sweetened herbal FMB; MF-HFMB, monk fruit-sweetened herbal FMB; C-FMB, control FMB.

3.6 Starter-culture populations in vitamin D_3-fortified herbal FMBs

Presumptive lactobacilli counts were statistically comparable among S-HFMB, MF-HFMB and C-FMB, ranging from 8.868 to 8.907 $\log_{10}$ CFU mL^{-1} ($p > 0.05$; Table 7). The incorporation of *Shatavari* and *Ashwagandha*, vitamin D_3 fortification and the type of sweetener did not significantly affect the presumptive lactobacilli counts. Milk provides lactose, peptides and minerals that support yoghurt-culture growth, while metabolic cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* can enhance the growth and acidification performance of both organisms (Ge et al. 2024).

In contrast, presumptive streptococci counts were significantly lower in S-HFMB than in MF-HFMB and C-FMB. Because S-HFMB and MF-HFMB contained the same botanical blend, the difference was more likely associated with the sweetener or resulting matrix properties than with botanical incorporation alone. Allulose can modify the metabolic activity of dairy lactic acid bacteria, although its effects are strain dependent and may not correspond to changes in cell growth (Kimoto-Nira et al. 2017). Similarly, monk fruit-based sweeteners have been reported to influence *Streptococcus thermophilus* populations in fermented milk, with responses depending on sweetener concentration and milk-matrix composition (Buchilina and Aryana 2021).

Table 7 Presumptive lactobacilli and streptococci counts in vitamin D_3-fortified herbal FMBs

Formulation	Presumptive lactobacilli count ($\log_{10}$ CFU mL^{-1})	Presumptive streptococci count ($\log_{10}$ CFU mL^{-1})

S-HFMB	8.868 ± 0.025[a]	9.200 ± 0.058[b]
MF-HFMB	8.878 ± 0.025[a]	9.430 ± 0.078[a]
C-FMB	8.907 ± 0.033[a]	9.490 ± 0.082[a]

Values are expressed as mean ± standard deviation. Within each microbial group, means bearing different lowercase superscripts differ significantly among formulations according to one-way analysis of variance followed by Tukey's multiple-comparison test ($p < 0.05$); S-HFMB, sucrose-sweetened herbal FMB; MF-HFMB, monk fruit-sweetened herbal FMB; C-FMB, control FMB; CFU, colony-forming units.

4. CONCLUSION

Response surface modelling established 0.35% Shatavari, 0.35% Ashwagandha and 12.50% sucrose as the optimum formulation (desirability = 0.96). Complete sucrose replacement with 12.5% commercial monk fruit-based bulk sweetener produced a highly acceptable beverage. Compared with the non-herbal control, the optimised beverage exhibited higher apparent viscosity (22.10 vs 20.40 mPa·s), DPPH radical-scavenging activity (0.92 vs 0.41 mmol TE L^{-1}) and *in vitro* antibacterial activity (19.63 vs 12.27 mm), while maintaining high presumptive lactobacilli and streptococci counts. Under the conditions tested, botanical incorporation and complete sucrose replacement did not prevent high sensory acceptability or high presumptive starter-group counts. However, the observed functional responses cannot be attributed specifically to individual botanical constituents, mogrosides or the sweetener carrier. Vitamin D_3 stability and bioaccessibility, phytochemical composition, refrigerated-storage stability and pilot-scale reproducibility require further investigation before commercial application.

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1011 **CONFLICT OF INTEREST**

1012 The authors declare that they have no conflicts of interest.

1013 **DATA AVAILABILITY STATEMENT**

1014 The data supporting the findings of this study are available from the corresponding author upon
1015 reasonable request.

1016 **Human ethics and ethics approval**

The sensory evaluation was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Research Committee of the College of Dairy Science, Kamdhenu University, Amreli, Gujarat, India.

Consent to participate

Informed consent was obtained from all participants before sensory evaluation. Participation was voluntary, and participants were informed of the study procedures and their right to withdraw at any time without penalty.